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Haworth, Jennifer A

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FROM ORTHODONTIC SEPARATORS TO THE HEART: A LABORATORY-BASED STUDY OF ORAL STREPTOCOCCAL BLOOD SURVIVAL MECHANISMS

Jennifer Ann Haworth

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Doctorate of Dental Surgery in the Faculty of Health Sciences

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The association between dental procedures, oral bacteria and infective endocarditis (IE) is contentious, particularly following changes in UK guidance on antibiotic prophylaxis in recent years. Although IE in children is rare, the disease carries a high risk of mortality and morbidity, and several cases of orthodontic-induced IE have been reported. Evidence suggests that orthodontic separator placement induces bacteraemia with oral streptococci, potential causative agents in IE. However, a detailed understanding of how such bacteria survive in blood is currently lacking.

This study aimed to determine the blood survival mechanisms of oral streptococci commonly implicated in orthodontic separator placement. In particular, the role of bacterial surface determinants and evasion of complement-mediated killing in promoting streptococcal survival in blood were investigated.

This work identified that bacterial blood survival rates are species- and strain-dependent, with a general correlation between IE-associated species and persistence in blood. IE pathogen *Streptococcus gordonii* surface proteins PadA and Hsa both bound Factor H and vitronectin, indicating two mechanisms by which *S. gordonii* may evade the host immune system.

Better understanding of mechanisms for streptococcal survival in the bloodstream, such as those identified in this project, could identify novel targets for the prevention or treatment of IE in the future.

Acknowledgements

I would like to thank Dr Angela Nobbs and Professor Tony Ireland for their supervision and generosity with their time during this project. My thanks also extend to all the volunteer blood donors in this project and to Helen Foskett, for co-ordinating the donations and taking the blood.

Dr Sylviane Yoba helped me extensively with several laboratory techniques. Dr Jane Brittan, Dr Lindsay Dutton, Dr Nihal Bandara and Dr Sarah Garner were very kind in helping to set up experiments.

Specific acknowledgement goes to Dr Ang Nobbs, for generation or *S. gordonii* strains UB2870 Δ*padA*/pMSP-*padA*-His-term and *S. gordonii* UB2935 Δ*hsa/*pMSP-*hsa*-His-term, which secrete native forms of PadA or Hsa, respectively, carrying a C-terminal His6 tag ($PadA_{His6}$ or Hsa_{His6}). Dr Sylviane Yoba and Dr Jane Brittan generated and purified His-tagged proteins ($PadA_{His6}$ or Hsa_{His6}).

Finally, I would like to thank my husband, my family and Mrs Jenny Pulham for their support.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: Jennifer Haworth DATE: 24th May 2020

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aa	amino acid
ECM	extracellular matrix
FH	Factor H
GRAS	generally regarded as safe
HUVEC	human umbilical vein endothelial cell
MAC	membrane attack complex
NET	neutrophil extracellular trap
ROS	reactive oxygen species
SrtA	sortase A
VGS	viridans group streptococci
Vn	vitronectin
vWF	von Willebrand factor
WT	wild-type

1. Literature review

1.1. The role of oral bacteria in health and systemic disease

1.1.1. Historical perspective

The mouth is often described as a 'window' into the health of the body, but the link between oral health and systemic health is not new. The importance of good oral hygiene was recognised as early as the Ancient Greek civilisation (O'Reilly and Claffey, 2000), and the eradication of mouth infections in patients with rheumatic joint problems was reported to cure symptoms by Hippocrates (Mayo, 1922). Antonie van Leeuwenhoek, a 17th century Dutch scientist, was the first to link a lack of oral hygiene to an increase in microorganisms, which he named "animalcules" in dental plaque. He was also the first scientist to use a microscope and is often referred to as the 'Father of Oral Microbiology'.

William Hunter, a British surgeon, published a report in 1900 emphasising the role of oral sepsis as a cause of disease (Hunter, 1900). Removal of teeth was common in the first half of the 20th century due to the popularisation of this focal infection theory, where localised infections were thought to be responsible for the initiation of inflammatory diseases. Conservative treatment became more popular in the second half of the 20th century, enabling patients to retain their dentition for longer. A seminal paper was published in 1989, reporting that oral health was significantly worse in patients with myocardial infarction, after adjusting for risk factors (Mattila *et al.*, 1989).

The turn of the 21st century saw an increased recognition that the oral cavity can act as a source of pathogenic bacteria and their products.

1.1.2. The role of oral bacteria in health

The oral cavity is a highly specialised environment, comprising several different soft and hard tissues, operating under constantly changing physical and chemical conditions. As a consequence, there are a number of different habitats for microorganisms. An estimated 700 bacterial species can inhabit the oral cavity (Dewhirst *et al.*, 2010), with between 100 and 200 species typically found in any one individual. However, problems with cultivation mean that so far only around half of the potential species have been named or isolated. By living in communities, known as biofilms, bacteria are more likely to survive in the relatively hostile conditions of the oral environment.

A biofilm is a collection of microorganisms found typically at the interface between two phases, enclosed within an extracellular matrix (Flemming and Wingender, 2010). Dental plaque is a widely studied example of a biofilm and comprises microorganisms enveloped within a matrix consisting of salivary components, bacterially-derived polymers and food particles. Salivary pellicle is the film that coats tooth surfaces and soft tissues, and this is a critical substratum for colonising oral bacteria, such as streptococci. The stages of oral biofilm formation are summarised in Figure 1.1. Briefly, bacteria in a planktonic state interact with newly-formed salivary pellicle on tooth surfaces. These primary coloniser bacterial cells adhere and aggregate to form a

'linking film', which then serves as the foundation for biofilm development. Inter- and intraspecies binding between bacterial cells (coadhesion) leads to accretion of the biofilm, including the incorporation of Gram-negative and anaerobic microorganisms, with the potential for pathogenic effects. The bacteria now function as a community in a sessile state.

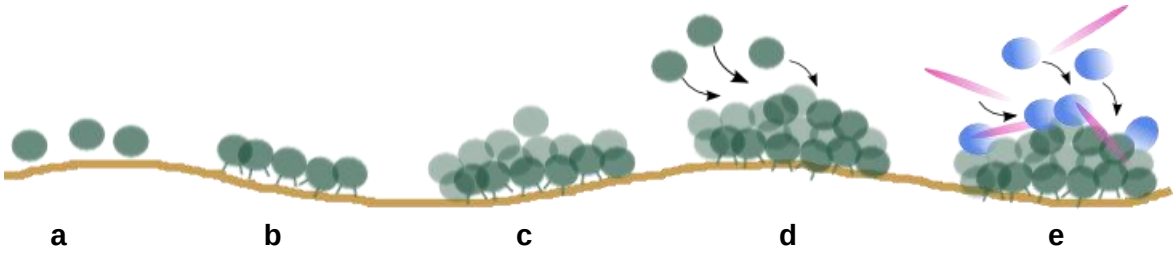

Figure 1.1 Stages in oral biofilm formation

a) Attachment of bacterial cells via non-specific forces
b) Higher affinity adhesion via specific receptors
c) Linking film formation
d) Planktonic cell recruitment
e) Maturation of the biofilm comprising multiple bacterial species

Microbial communities, known as microbiomes, are found in and on many parts of the body, and the microbial component of the human holobiont is substantial, equalling the number of human cells (Sender, Fuchs and Milo, 2016). There are many positive effects of host-microbiome symbiosis, including anti-inflammatory properties, anti-oxidant activity, maintenance of a healthy digestive tract and resistance to colonisation by pathogens (Kilian *et al.*, 2016). The role of the oral microbiome in health and disease has been studied widely in recent years, and when resident species in the oral cavity

are in equilibrium with each other and with the host, a symbiotic or health state exists. The role of early colonisers is important here, with formation of a stable microbial community that inhibits colonisation by incoming pathogens. This concept is known as colonisation resistance, and was first described in the 1950s with regards to antibiotic-mediated disruption of intestinal tract microbial communities (Bohnhoff, Drake and Miller, 1954). A complex balance of microbe-host and microbe-microbe interactions is required to mediate protection against invading pathogens throughout life in a healthy host (Lawley and Walker, 2013). Other changes in the local environment that can disturb the diversity and relative proportions of species within a community, leading to a so-called dysbiotic microbiome, include changes in oral hygiene, diet, smoking and diseases such as diabetes (Kilian *et al.*, 2016).

A recent systematic review found the use of oral probiotics may promote maintenance of good oral health by reducing the microbial load of oral pathogens (Seminario-Amez *et al.*, 2017). More personalised treatment has also been proposed for the clinical management of diseases such as caries and periodontal disease, through the use of oral microbiome transplants (Allaker and Stephen, 2017).

1.1.3. The role of oral bacteria in systemic disease

Many associations between oral and systemic diseases have been found. These include heart disease (Mustapha *et al.*, 2007), ventilator-associated pneumonia (Heo *et al.*, 2008), adverse pregnancy outcome (Han, 2011), diabetes mellitus (Molina *et al.*, 2016), rheumatoid arthritis (Kaur *et al.*, 2014), inflammatory bowel disease (Strauss *et al.*,

2011), colorectal cancer (Castellarin *et al.*, 2012) and liver disease (Han, Sun and Yang, 2016). Many of these diseases share risk factors such as age, male gender, smoking, alcohol, hypertension, social isolation and stress (Beck *et al.*, 1996). However, while associations clearly exist, causation between oral bacteria and systemic disease has often been more difficult to determine. Three mechanisms for the link between oral microbes and systemic diseases have been suggested (Thoden van Velzen, Abraham-Inpijn and Moorer, 1984). These are described below and in Figure 1.2.

1) Bacteraemia and extra-oral site colonisation

The entry of oral bacteria into the blood may allow colonisation at sites away from the oral cavity. Normal physiological activities, such as chewing and tooth-brushing, can result in bacteraemia. This is in fact an old concept as described by Elliott in 1939:

> "*bacteria may also gain admission to the blood-stream in such cases irrespective of operative procedures and probably as the result, in many instances, of minor degrees of gum injury such as is produced by biting on a loose tooth"* (Elliott, 1939)

It is thought that cumulative levels of bacteraemia are higher during day-to-day activities, including flossing or chewing, than after a single tooth extraction (Que and Moreillon, 2011). Both cardiovascular and non-cardiovascular sites are often found to be colonised by oral microorganisms (Han and Wang, 2013). These microorganisms have adapted to survive at such extra-oral sites, escaping

host surveillance and developing mechanisms to persist at these sites (Han and Wang, 2013).

2) **Systemic injury from bacterial toxins**

Toxin production by oral bacteria (e.g. endotoxins) can directly damage the host. Lipopolysaccharide (LPS), an endotoxin found in the outer membrane of Gram-negative bacteria such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* is associated with cerebral infarction, adverse pregnancy outcome and idiopathic trigeminal neuralgia (Li *et al.*, 2000b), as well as fever, toxic shock and apoptosis (Lamont, Hajishengallis and Jenkinson, 2014).

3) **Systemic inflammation as a result of immunological response to oral microbes**

The deposition of immune complexes, which form when soluble antigens from oral bacteria react with circulating antibodies, can lead to chronic or acute inflammation. Levels of inflammatory mediators, such as prostaglandin E_2, interleukin-1β and tumour necrosis factor α, become raised. Several conditions have been associated with systemic inflammation caused by oral microorganisms, including inflammatory bowel disease (Van Dyke *et al.*, 1986), uveitis, Behçet's syndrome and chronic urticaria (Li *et al.*, 2000b).

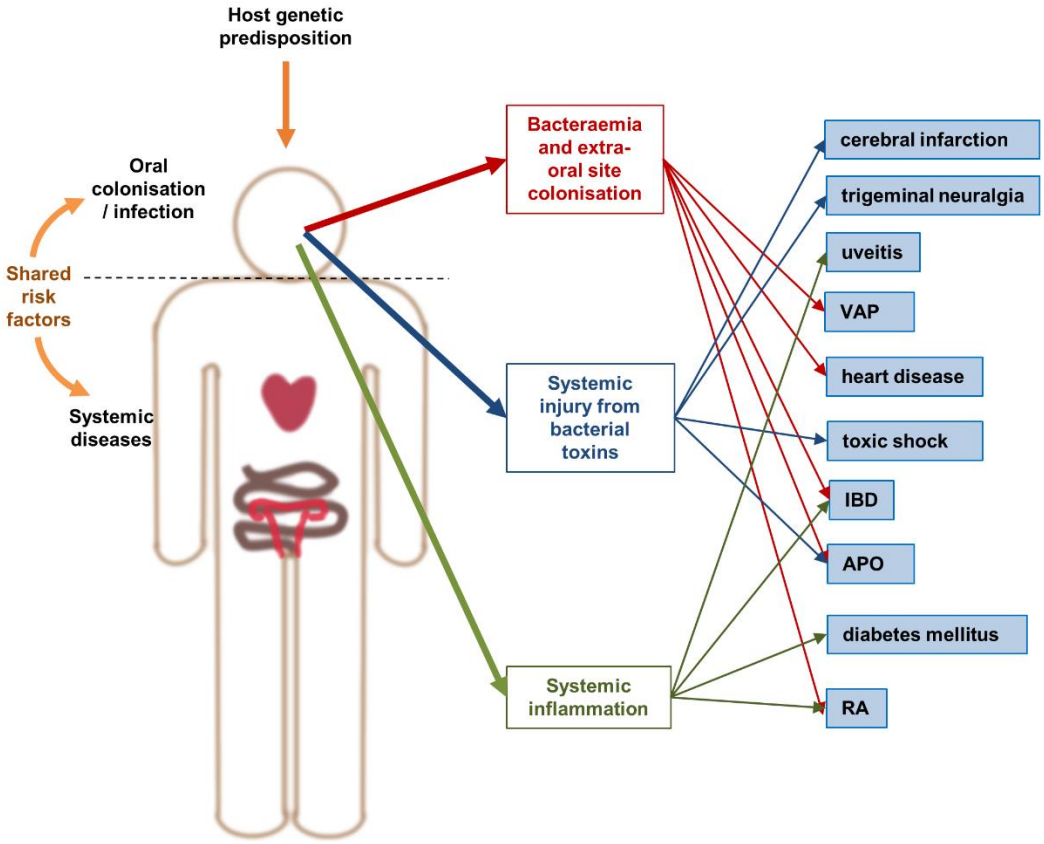

Figure 1.2 Mechanisms linking oral bacteria and systemic disease (adapted from Haworth et al., 2017b).

VAP = ventilator-associated pneumonia; IBD = inflammatory bowel disease; APO = adverse pregnancy outcome; RA = rheumatoid arthritis

1.2. Effects of orthodontic treatment on the oral microbiome

It has been recognised for many decades that wearing orthodontic appliances increases the likelihood of dental plaque retention. In Bloom and Brown's study of 1964, there was an increase in several microbial populations following the placement of orthodontic appliances, with the microbial increase proportional to the number of orthodontic bands in the mouth. Research regarding the effects of wearing fixed orthodontic appliances has shown that the bracket base is a critical site for plaque accumulation, particularly where excess bonding composite is present (Sukontapatipark *et al.*, 2001). The relative impact of bonds versus bands in fixed orthodontic appliances has also been the subject of investigation over the years. Ireland *et al.* (2014) showed that molar bonds are more likely to elicit changes towards a microbiota with raised disease potential, compared to molar bands. This is in contrast to evidence from Boyd and Baumrind (1992), where banded molars showed increased plaque accumulation compared to bonded molars.

The formation of white spot lesions or decalcification around fixed orthodontic appliances is common, although the reported incidence varies widely from 50% (Gorelick, Geiger and Gwinnett, 1982) to 97% (Boersma *et al.*, 2005). Decalcification often occurs early in treatment, with a sharp increase in the number of white spot lesions during the first 6 months of treatment and a slower rate of increase in the following months (Tufekci *et al.*, 2011). As in any carious lesion, there is an ecological shift in the dental biofilm towards a bacterial population that is aciduric. Balenseifen and Madonia (1970) reported a rise in *Lactobacillus* content of plaque after

orthodontic band placement, with concurrent increases in *Streptococcus salivarius* and *Streptococcus mitis*. Orthodontic band placement changes the chemical composition in the oral cavity, with decreased pH, calcium ion and phosphate ion levels, favouring more acidogenic plaque (Chatterjee and Kleinberg, 1979). It is also likely that the materials from which fixed appliances are made alter the plaque composition. Cariogenic streptococci have been found to adhere more readily to plastic rather than monocrystalline brackets (Ahn, Lim and Lee, 2007). *Streptococcus mutans* is the classic microorganism associated with caries, but recent evidence has suggested that there may be a role for *S. gordonii* in enamel demineralisation around orthodontic appliances (Sadeq *et al.*, 2015).

There is conflicting evidence regarding the long-term effects of orthodontic treatment on the microbiota, particularly in relation to periodontal health. Periodontal pathogens, such as *Selenomonas* species and *P. gingivalis*, *Tannerella forsythia* and *Eubacterium nodatum* have been associated with fixed appliance treatment (Ireland *et al.*, 2014; Koopman *et al.*, 2015). A study by Van Gastel and Quirynen (2008) reported that microbial and clinical periodontal changes occurred in patients undergoing fixed appliance treatment, with the changes occurring more quickly at bonded sites compared to banded sites. The picture is further complicated by the differences that occur between adolescents and adults during treatment. Adolescents with fixed appliances show more gingival inflammation and plaque accumulation than adults (Boyd and Baumrind, 1992). Others have reported that there is no increased risk for high levels of periodontal pathogens from fixed appliance therapy (Thornberg *et al.*,

2009). Nevertheless, Soro *et al.* (2014) identified the candidate division TM7 microorganism associated with orthodontic bonds. This microorganism is thought to have a preference for the subgingival mature plaque associated with periodontal disease (Brinig *et al.*, 2003) and is now known to establish a long-term parasitic association with bacterial host *Actinomyces odontolyticus (*Bor *et al.,* 2018*)*.

The oral microbial community shows remarkable resilience to the use of orthodontic appliances (Koopman *et al.*, 2015), although there is evidence that even 1 year after orthodontic treatment is completed, restoration to pre-treatment microbiota fails to occur (Ireland *et al.*, 2014). The long-term effects of orthodontic treatment on the oral microbiome are therefore not fully understood.

1.3. Oral bacteria and heart disease

Cardiovascular disease continues to be a major burden in the UK (Bhatnagar *et al.*, 2015), despite widespread use of anti-cholesterol treatment and promotion of healthy lifestyles. Microbial infection is thought to be a risk factor for cardiovascular disease, and bacteraemia incidence is rising. This may be due to increasing numbers of interventional procedures (Fitzgerald, Foster and Cox, 2006). Oral streptococci, commonly implicated in infective endocarditis (IE), can be isolated from blood culture following physiological activities and this may be as important to disease pathogenesis as dentistry-induced bacteraemia (Roberts, 1999). Others have calculated that cumulative levels of bacteraemia are higher during day-to-day activities, such as chewing or flossing, than after a single tooth extraction (Que and Moreillon, 2011).

Periodontal disease is thought to be associated with atherosclerosis, the result of artery wall thickening with a concurrent inflammatory component (Ross, 1999), and periodontal pathogens can be found in atherosclerotic plaques (Haraszthy *et al.*, 2000). Increased serum antibody levels to oral anaerobes are found in some patients with acute myocardial infarction (MI) (Burazor and Vojdani, 2014). Stroke is the result of thrombotic ischaemia or haemorrhage, and evidence supports associations between oral infections and stroke (Syrjanen *et al.*, 1989; Hashemipour *et al.*, 2013).

1.3.1. Infective endocarditis

IE is characterised by the formation of infected vegetations on the endocardial surface of the heart, with the heart valve surfaces commonly implicated (Figure 1.3). These vegetations comprise bacteria, fibrin and platelets. The morbidity and mortality rates of IE are high, with between 15% and 20% of endocarditis patients dying during their initial hospital admission (Thornhill *et al.*, 2016b). Mortality rates are high because the sequelae of IE, if treatment is not successful, include embolism, valve dysfunction with subsequent heart failure or uncontrolled infection (Cahill and Prendergast, 2016). In fact, it has been suggested that IE should be viewed as a form of systemic sepsis (Wallace *et al.*, 2002). Sepsis occurs when there is a deregulated response to infection, leading to systemic inflammation (Shannon, 2015).

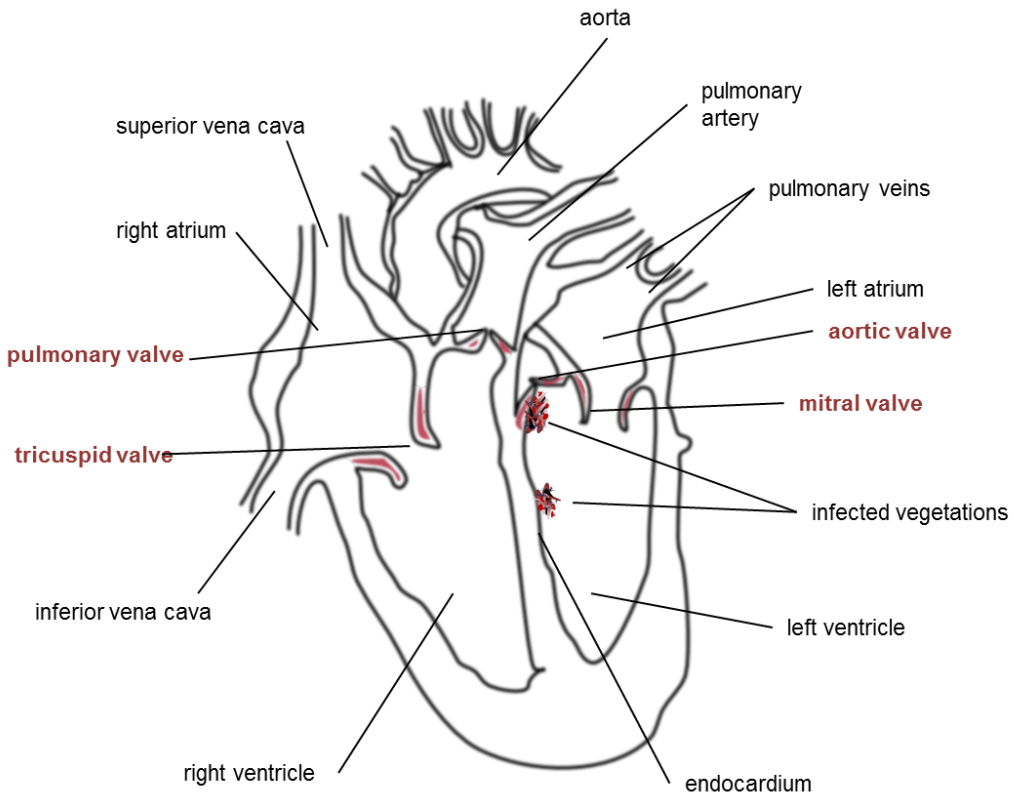

Figure 1.3 The heart with infected vegetations.

The vegetations are shown and the valve sites highlighted.

1.3.1.1. Epidemiology

The incidence of IE has been reported as 1-5 per 100,000 in the American and European general populations per year (Tleyjeh *et al.*, 2007). In the UK there are over 2000 cases annually (Thornhill *et al.*, 2016b), and evidence suggests that the incidence of IE has risen in recent years following the withdrawal of prescriptions for antibiotic prophylaxis prior to invasive dental procedures. This was in response to revised guidelines by the National Institute for Health and Care Excellence (NICE) (Dayer *et al.*, 2015).

Left-sided native valve endocarditis is the most common form of IE and this tends to occur in patients with congenital heart defects or damaged heart surfaces from rheumatic fever. The most severe form of IE is left-sided prosthetic valve endocarditis. This can be categorised as early, often due to surgery-related microbes such as staphylococci, or late, occurring more than 12 months after surgery and often connected with oral streptococci or Gram-negative microorganisms. Risk groups that have emerged in recent years include haemodialysis patients, patients with valve sclerosis and IV-drug abusers (Fitzgerald, Foster and Cox, 2006). The latter are particularly prone to right-sided IE, with the causative agents often arising from the skin, such as staphylococci. A recent study by Rosenthal *et al.* (2016) reported that injection-drug users are at risk of recurrent IE, high rates of readmission and death rates in the region of 25%. Healthcare-associated endocarditis is the final category of IE and this can occur in both nosocomial and non-nosocomial settings.

IE is a rare diagnosis in children. A district general hospital-based paediatrician is only likely to see 1-2 cases per year in the UK (Singh and Ganjoo, 2017). Despite this, paediatricians often have a high index of clinical suspicion regarding IE because of the associated high morbidity and mortality. Diagnosis is difficult because the classical signs of endocarditis are often absent in children. At risk children have historically been those who have suffered from rheumatic heart disease. Currently the main risk groups are children with congenital heart defects, intra-cardiac repairs and central venous catheters (Singh and Ganjoo, 2017). However, 10% of children have no identifiable risk

factors. Staphylococci are reported to account for 43% of IE cases in children, with streptococci accounting for 39% of cases (Grattan *et al.*, 2015).

1.3.1.2. Pathogenesis

Three critical elements are required for IE pathogenesis (Sullam, Drake and Sande, 1985). Firstly, the cardiac valve surface must be altered to allow bacterial adherence. For example, trauma can alter the endothelial surface, leading to platelet and fibrin deposition and surface disruption. Secondly, circulating bacteria adhere to the altered valve surface, with some bacteria adhering to the platelet-fibrin matrix on the surface more readily than others. Thirdly, the adherent bacteria must survive and resist host immune responses, such as the complement cascade, with propagation of the infected vegetation. Activation of the clotting cascade results in growth of a platelet-fibrin clot over the bacteria. The infected vegetation can then break away and seed at other susceptible sites (Sullam, Drake and Sande, 1985).

The importance of bacteria-platelet interactions has emerged in recent years, as well as the complexities of the non-thrombotic roles of platelets. Bacteria can trigger platelet adhesion, aggregation, spreading and thrombus formation, and it is hypothesised that platelets can restrict the spread of infection and control the immune response through the release of contents from their intracellular granules. Alternatively, bacteria-platelet interactions could be regarded as a mechanism by which bacteria can protect themselves via a platelet coating from immune cell attack

or antibiotic penetration (Kerrigan, 2015). Streptococcal-platelet interactions will be described in more detail in section 1.5.3.

1.3.1.3. Microbiology of endocarditis

Together, enterococci, streptococci and *Staphylococcus aureus* are responsible for more than 80% of cases of IE (Fitzgerald, Foster and Cox, 2006).

Enterococci, most frequently *Enterococcus faecalis*, cause 5-20% of cases of IE. The most frequent source of infection is the genitourinary tract, and both normal and previously damaged cardiac valves can be affected (Megran, 1992). *E. faecalis* has been shown to interact with platelets via platelet receptor FcγRIIa and host IgG (Rasmussen *et al.*, 2010).

Oral streptococci are responsible for 35-45% of cases of IE (Dayer *et al.*, 2015). They are common causes of both native valve and late prosthetic valve endocarditis. Their role in IE will be covered in more detail below.

S. aureus is a facultative anaerobe that can cause a range of diseases. IE in IV drug users, prosthetic valve IE and healthcare-related IE are particularly associated with *S. aureus* (Que and Moreillon, 2011). The increase in these risk groups has led to an increased incidence of staphylococcal endocarditis. Platelets are thought to interact with staphylococci via three main mechanisms: 1) through the release of toxins such as α-toxin; 2) as a result of cell-wall protein-mediated platelet activation; and 3) through platelet internalisation of *S. aureus* cells (Kerrigan and Cox, 2012).

Although rare, IE can be caused by fungal species and these are particularly important in cases of early prosthetic valve endocarditis (Que and Moreillon, 2011). Candidal endocarditis has a particularly high mortality rate and is increasingly becoming a healthcare-associated disease (Mohammadi, Mohammadi and Forrest, 2013). *Candida* species are known to cause platelet aggregation (Willcox *et al.*, 1998).

1.4. Oral streptococci

Streptococci are Gram-positive bacteria that grow in pairs or chains. They are generally facultative anaerobes and more than 100 species have been recognised. Six taxonomic groupings have been described: pyogenic, salivarius, bovis, mutans, mitis and anginosus (Nobbs, Lamont and Jenkinson, 2009). Streptococci within the oral cavity are often also collectively referred to as viridans group streptococci (VGS). This is due to the alpha haemolysis that occurs when these streptococci are grown on blood agar, leading to a green colouration of the agar; “viridans” means “greening” in Latin. VGS are early colonisers of the oral cavity and play an important role in colonisation resistance. They are generally commensal microorganisms, but they can become pathogenic when they enter the bloodstream. They have many surface proteins capable of binding to oral surfaces and to endothelial tissue, platelets and extracellular matrix (Jakubovics *et al.*, 2005a). These protein adhesins have been grouped into families based on similarities in sequences or structure (Nobbs, Lamont and Jenkinson, 2009). Selected adhesin families are discussed below.

Important families of streptococcal adhesins are shown in Table 1.1. Most of the adhesins named are anchored to the cell wall with a C-terminal motif (LPxTz). This motif is generally followed by a hydrophobic region and a charged tail (Nobbs, Lamont and Jenkinson, 2009). The motif is recognised by transpeptidase sortase A, which cleaves the adhesin at this site and facilitates its attachment to the bacterial cell wall.

Table 1.1 Important oral streptococcal surface protein families

Streptococcal surface protein family	**Surface protein example**	**Species**	**Reference**
Serine-rich repeat	SrpA	*Streptococcus sanguinis*	Plummer *et al.* (2005)
	Fap1	*Streptococcus parasanguinis*	Wu *et al.* (1998)
	Hsa/GspB	*S. gordonii*	Jakubovics *et al.* (2005)
Antigen I/II	SpaP	*S. mutans*	Kelly *et al.* (1989)
	SspA/SspB	*S. gordonii*	Jenkinson and Demuth (1997)
Fibrils	CshA, CshB	*S. gordonii*	McNab *et al.* (1999), McNab *et al.* (1994), Elliott *et al.* (2003)
	CshA-like protein	*S. sanguinis*	Elliott *et al.* (2003)
	CshA-like protein	*Streptococcus oralis*	Elliott *et al.* (2003)
Pili	PilA, PilB, PilC	*S. sanguinis*	Okahashi *et al.* (2010)
	PI-2 pili	*S. mitis*	Zahner *et al*. (2011)
Other	PadA	*S. gordonii*	Petersen *et al.* (2010), Keane *et al.* (2013), Haworth *et al.* (2017)

The serine-rich repeat (SRR) polypeptides are glycosylated and contain multiple blocks of serine-rich amino acid residues. Their N-terminal domain binds various substrata (Takahashi *et al.*, 2002). The surface protein Hsa, present on *S. gordonii* DL1, recognises receptors on platelets, epithelial cells, polymorphonuclear leucocytes and immobilised

glycoprotein-340 (gp340) (Jakubovics *et al.*, 2005a). Hsa orthologues are present across oral streptococcal species and are known to make significant contributions to virulence in animal models of IE (Xiong *et al.*, 2008). Hsa has recently been shown to act cooperatively with another *S. gordonii* surface protein, PadA, in binding to platelets, vitronectin (Vn), salivary pellicle and in biofilm formation (Haworth *et al.*, 2017a).

The antigen I/II (AgI/II) family polypeptides are found across a wide range of oral streptococci (Jenkinson and Demuth, 1997), as well as in *Streptococcus suis*, *Streptococcus agalactiae* and *Streptococcus pyogenes* (Brady *et al.*, 2010). These proteins form fibrillar structures and bind to type I collagen, fibronectin and epithelial cells, as well as mediating interactions with other microorganisms such as *Actinomyces oris* and *P. gingivalis* (Jakubovics *et al.*, 2005b). SspA and SspB, present on *S. gordonii*, induce platelet aggregation (Kerrigan *et al.*, 2007).

Fibrils extend between 40 nm and 400 nm from the bacterial surface (Nobbs, Lamont and Jenkinson, 2009). They are fine structures, but evidence is limited regarding their function. *S. gordonii* fibril CshA is known to interact with fibronectin and other microorganisms (Wright *et al.*, 2013; Back *et al.*, 2017).

Pili are 3-10 nm in diameter and can extend up to 3 µm from the bacterial surface (Nobbs, Lamont and Jenkinson, 2009). They are generally found in pathogenic streptococci, such as *Streptococcus pneumoniae* and *S. pyogenes*. Pili bind gp340 and epithelial tissues (Crotty Alexander *et al.*, 2010; Brittan and Nobbs, 2015). They also

play a role in DNA-mediated transformation in *S. pneumoniae* (Laurenceau *et al.*, 2013).

PadA is a large cell wall-anchored protein expressed on the surface of *S. gordonii*. The platelet fibrinogen receptor αIIbβ3 binds common integrin-recognition motifs within the N-terminal region of PadA (Keane *et al.*, 2013). The NGR motif within PadA is essential for interaction of PadA with activated platelets (Haworth *et al.*, 2017a). This mirrors the importance of the NGR motif in fibrinogen for interaction with platelets. The NGR motif is present in the D domain of fibrinogen on each of the β and γ chains and plays an important role in binding αIIbβ3 (Moriarty *et al.*, 2015).

1.5. Oral streptococcal virulence mechanisms in IE

Most oral cavity microorganisms are commensal, but many are also opportunistic pathogens, capable of causing major infections under certain conditions. Oral streptococci can cause disease when they enter the bloodstream, such as IE (Douglas *et al.*, 1993).

1.5.1. Bacterial-endothelial cell interactions

Bacteria need to adhere to endothelial surface components of the cardiac valve before they can invade and/or colonise (Vercellotti *et al.*, 1984). Often, some alteration of the endocardial surface is required to allow it to be colonised (Kielhofner and Hamill, 1989), although IE does also occur in patients with no known endocardial surface changes. In fact, acute endocarditis occurs on normal heart valves in at least 50% of cases (Ogawa *et al.*, 1985). Bacteria, including *S. aureus* and VGS, have been found to

attach to cardiac endothelial cells *in vitro* (Gould *et al.*, 1975). In addition, *S. aureus* invades and survives within endothelial cells, causing persistent infection and endothelial destruction (Menzies and Kourteva, 1998). Viridans streptococci demonstrate lower adherence to endothelial surfaces than *S. aureus* (Ogawa *et al.*, 1985) and there is evidence that VGS can invade cardiac endothelium (Stinson, Alder and Kumar, 2003). *S. mutans* has been shown to bind *ex vivo* human heart tissue (Freires *et al.*, 2017). Although *S. gordonii* has been investigated for its ability to bind to human umbilical vein endothelial cells (HUVECs) *in vitro* (Vacca-Smith *et al.*, 1994), very little is known regarding the mechanism of attachment and the endothelial cell ligands *in vivo*. Glucosyltransferase, Hsa, CshA and CshB have been suggested as possible mediators of attachment of *S. gordonii* to HUVECs *in vitro* (Stinson, Alder and Kumar, 2003).

1.5.2. Bacteria-extracellular matrix interactions

The extracellular matrix (ECM) beneath the endothelium is composed of an intricate mixture of macromolecules, including several different types of collagen, laminin, fibronectin, Vn, proteoglycans and glycosaminoglycans (Zhao *et al.*, 2016). As well as its structural role, the ECM is thought to be implicated in endothelial cell migration, proliferation and differentiation (Mintz, 2004). Physiologically, ECM proteins are important in the process of haemostasis (Figure 1.4). Von Willebrand factor (vWF), collagen and tissue factor are normally contained within the subendothelium. When a blood vessel is traumatised, these ECM proteins trigger primary haemostasis, where a platelet plug forms, quickly sealing the circulatory system, stopping further blood loss

and preventing pathogens from entering the circulation (Broos *et al.*, 2011). After blood vessel injury, vWF, immobilised on collagen in the subendothelial tissues, interacts with the GPIb platelet receptor, leading to platelet rolling, slowing the platelet (Clemetson, 2012). When the platelet has slowed sufficiently, GPVI and GPIaIIa bind to collagen, leading to firm adhesion, along with GPIIbIIIa binding to vWF (Broos *et al.*, 2011).

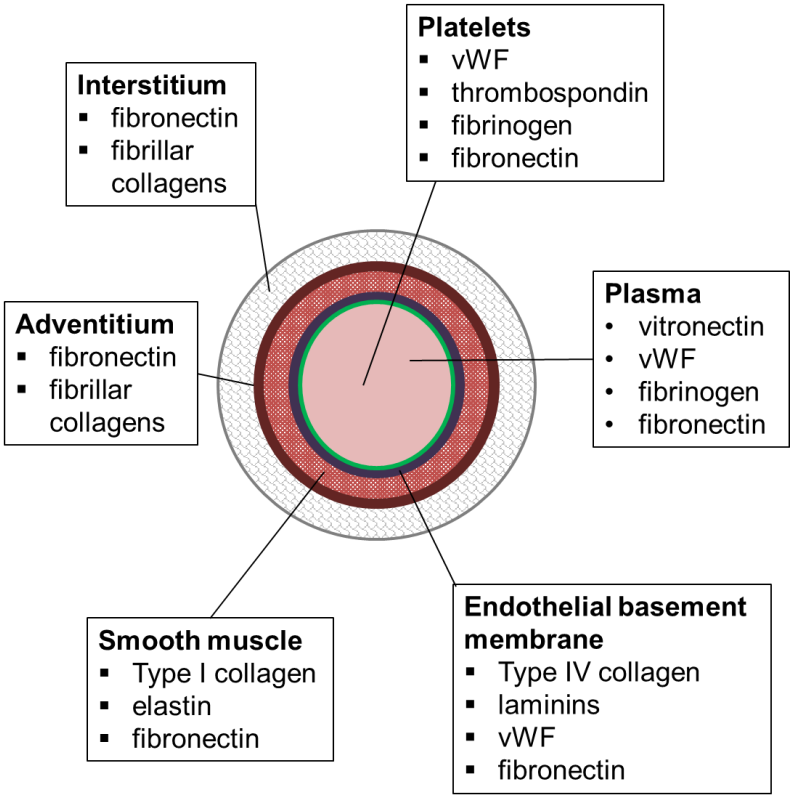

Figure 1.4 Cross-section of a blood vessel showing ECM proteins important for haemostasis. Adapted from Bergmeier and Hynes (2012).

Vessel damage may expose the wide variety of ECM proteins shown in Figure 1.4. These may then play an important role in bacterial attachment at damaged cardiac endothelial sites. Fibronectin and Vn are both highly expressed on cardiac endothelium (Vercellotti *et al.*, 1984). Other ECM proteins that will not be discussed in detail include collagen, fibrinogen, vWF, laminin, plasminogen and thrombospondin.

Fibronectin is secreted by hepatocytes and fibroblasts. It is composed of 250 kDa subunits, with a large number of variants, generating molecules with different cell-adhesive or ligand-binding properties (Schwarzbauer and DeSimone, 2011). Fibronectin can be classified in 2 forms according to its solubility (Pankov and Yamada, 2002): insoluble fibronectin is produced by fibroblasts and endothelial cells (Mao and Schwarzbauer, 2005) and is assembled into a matrix when deposited into the ECM; soluble fibronectin is produced by hepatocytes and circulates in the plasma and is stored in platelet α-granules.

Adhesion to fibronectin is often an important element of bacterial virulence. Fibronectin-binding proteins of Gram-positive cocci allow the bacteria to adhere to fibronectin-containing surfaces, and may also mediate internalisation of bacteria into epithelial and endothelial cells (Schwarz-Linek, Höök and Potts, 2006). *S. gordonii* adhesins known to be implicated in fibronectin adhesion include CshA/CshB (McNab *et al.*, 1996), Hsa (Takahashi *et al.*, 2002), SspA and SspB (Giomarelli *et al.*, 2006; Nobbs *et al.*, 2007a; Jakubovics *et al.*, 2009) and PadA (Haworth *et al.*, 2017a). Fibronectin binding is likely to be critical in the pathogenesis of IE, since fibronectin is exposed

when the endothelium is injured and binds platelets and fibrin, therefore contributing to surface thrombogenicity (Hamill, 1987).

Vn is contained in plasma and in the α-granules of platelets. It is also present in a variety of epithelial and endothelial tissues. It can play a role in stabilising thrombus formation and can form complexes with plasminogen activator inhibitor-1, thereby acting as a physiological inhibitor of active thrombin. A number of Gram-positive and Gram-negative bacteria have been shown to bind Vn, which can then act as a bridge between bacteria and epithelial cells, facilitating adhesion (Singh, Su and Riesbeck, 2010). There is evidence that Vn is also important for streptococcal adherence to HUVECs (Valentin-Weigand *et al.*, 1988). Alongside adhesion, bacterial bound Vn has been shown to confer serum resistance by blocking components of the complement system and preventing formation of the membrane attack complex (Singh, Su and Riesbeck, 2010). Recently, *S. gordonii* was shown to bind Vn via Hsa and PadA (Haworth *et al.*, 2017a). This could likely augment the systemic survival of this bacterium.

1.5.3. Bacteria-platelet interactions

The primary role of platelets in the bloodstream is haemostasis after blood vessel injury. However, platelets are increasingly recognised as an important part of the innate immune response. They are ideally suited as immune response effectors because they are continuously in circulation. Nevertheless, platelets are thought to have both protective and adverse effects in infectious disease (Morrell *et al.*, 2014).

Platelets bind and surround bacteria in the bloodstream, effectively hiding the invading bacteria from host immune components such as phagocytes. This has resulted in platelets being described as “Trojan Horse” carriers of bacteria in the circulation (Deng *et al.*, 2014).

Three modes of bacteria-platelet interaction have been described (Kerrigan and Cox, 2012):

1) Direct binding of a bacterial surface component by a receptor on the platelet surface.
2) Indirect binding of a platelet receptor to bacteria through a plasma protein acting as a bridge, such as fibrinogen or complement.
3) Interaction of bacterial toxins with platelets.

A summary of these interactions is shown in Figure 1.5.

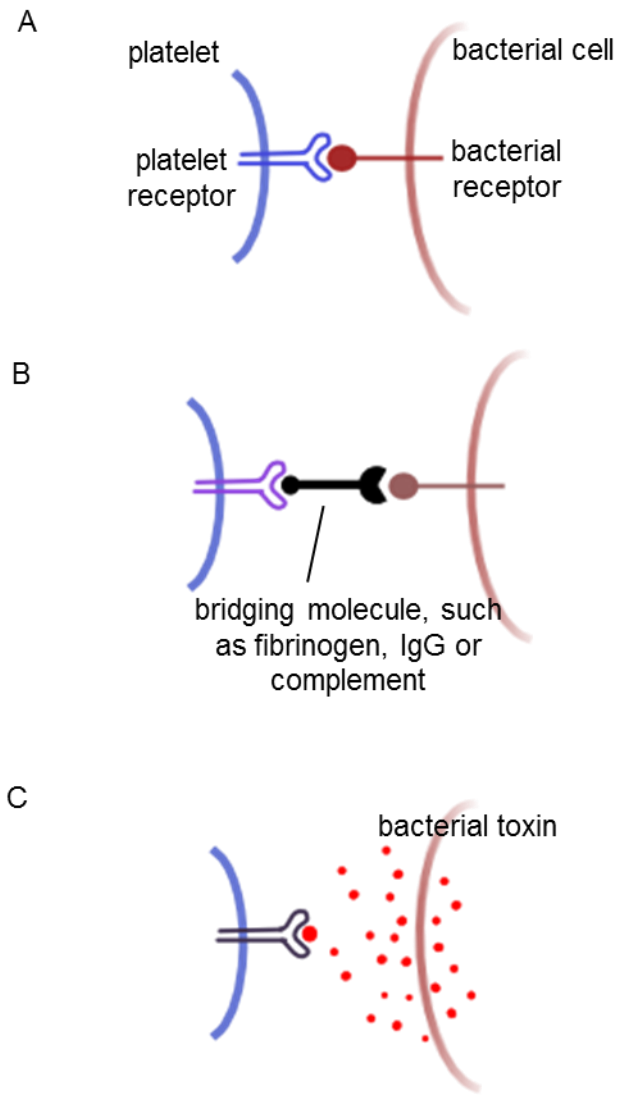

Figure 1.5 Interactions between platelets and bacteria.

A) Direct interaction
B) Indirect interaction
C) Bacterial toxin-mediated interaction

The interaction of bacteria and platelets in the bloodstream is likely to be critical for the formation of the infected vegetations in IE. In fact, Moreillon *et al.* (1988) reported that the ability of a streptococcal strain to adhere to a sterile vegetation (i.e. platelet and fibrin thrombus) is likely to be more important than the size of the bacteraemia. Oral streptococci selectively target platelets in whole blood over other blood components *in vitro* (Deng *et al.*, 2014).

Recent work has delineated the interactions of IE pathogen *S. gordonii* with human platelets. Hsa interacts with GPIb and αIIbβ3 on the platelet surface, capturing platelets under flow. Platelet intracellular signalling cascades are activated, including FcγRIIa phosphorylation. PadA then binds activated αIIbβ3, further amplifying signals leading to platelet shape change, thrombin production, coagulation and thrombus formation (Haworth *et al.*, 2017a). SspA and SspB also interact with an unknown receptor on the platelet surface, stimulating platelet aggregation (Kerrigan *et al.*, 2007). Proposed mechanisms for the pathogenesis of *S. gordonii* endocarditis are described in Figure 1.6.

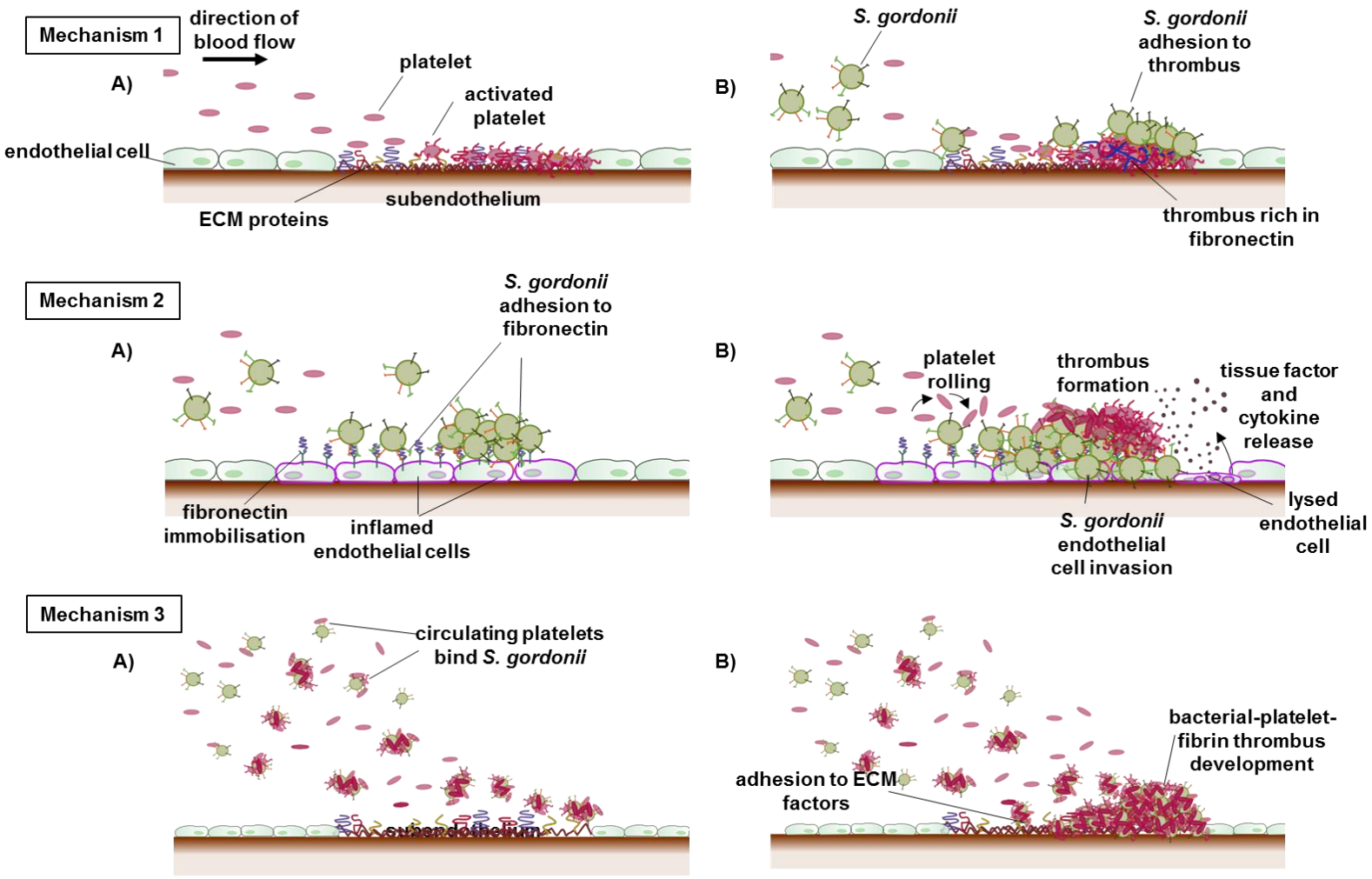

Figure 1.6 Three proposed models of *S. gordonii* IE pathogenesis (from J. Haworth PhD thesis).

Mechanism 1) Adhesion of *S. gordonii* to existing non-bacterial thrombotic endocarditis deposits. **A)** ECM is exposed in damaged heart tissue as a result of trauma or rheumatic fever. Platelets bind to exposed ECM proteins, such as collagen, vWF, Vn and fibronectin, forming a non-bacterial thrombotic endocarditis deposit. Fibronectin is abundant in these sterile vegetations. **B)** *S. gordonii* binds to immobilised fibronectin via PadA and Hsa, as well as CshA/CshB and SspA/SspB. Vn and vWF may also encourage *S. gordonii* binding to the vegetation via Hsa and PadA. Activated platelet surfaces present in the thrombus capture circulating *S. gordonii* cells via interactions of Hsa and PadA with GPIbα and GPIIbIIIa. PadA mediates shear resistance and Hsa and PadA may play a role in co-adhesion with other *S. gordonii* cells, contributing to growth of the vegetation.

Mechanism 2) Adhesion of *S. gordonii* to immobilised fibronectin on inflamed valvular endothelium and subsequent mimicking of the vessel wall.
A) Local inflammation of intact valvular endothelium results in integrin expression. Plasma fibronectin binds integrins and *S. gordonii* adheres to the immobilised fibronectin on the surface. **B)** Bacterial cells mimic host tissue and cause platelet rolling and subsequent platelet adhesion and activation. Subsequent invasion of endothelial cells by *S. gordonii* triggers tissue factor activity and cytokine release, encouraging further inflammation, coagulation and platelet clot formation. Lysis of endothelial cells may occur.

Mechanism 3) Transport of *S. gordonii* cells by platelets to damaged endocardium.
A) Circulating *S. gordonii* cells bind circulating platelets forming microthrombi. The Hsa-GPIbα interaction is hypothesised to be the predominant mechanism here, although other bacterial platelet-interactive proteins may play a role. The platelets are effectively acting as Trojan Horse carriers to the damaged heart valve sites. **B)** Both platelet and bacterial receptors facilitate binding to exposed ECM factors, such as fibronectin, collagen, Vn and vWF.

1.5.4. Bacteraemia and bacterial survival in blood

Bacteraemia can be defined as the presence of viable bacteria in the bloodstream (Christaki and Giamarellos-Bourboulis, 2014). The incidence of bloodstream infections has increased, with recent evidence from Switzerland showing a 14% increase over a 6 year period (Buetti *et al.*, 2017). For a bacteraemia to occur, microorganisms must first gain entry through either the skin or the mucosal surfaces of the oropharynx, gastrointestinal, genitourinary or respiratory tracts. The oral bacterial biofilm differs from other biofilms in that it is very close to a highly vascularised milieu. The subgingival epithelium, which is only 10 cell layers thick, can easily be disturbed, resulting in bacteraemia (Parahitiyawa *et al.*, 2009).

Neutrophils are one of the first and most important host defence mechanisms against invading microbes. Neutrophils migrate to the site of infection, with the recruitment mediated by chemoattractants such as interleukin-8, leukotriene B_4 and granulocyte chemotactic protein 2 (Christaki and Giamarellos-Bourboulis, 2014). Pattern recognition receptors are an important part of the innate immune system. They recognise common microbial structures known as pathogen-associated microbial patterns (PAMPs). Neutrophil recognition and phagocytosis of invading microorganisms is facilitated by complement receptors that bind to complement and antibody-coated microbes. Other cells capable of phagocytosis include tissue macrophages, dendritic cells and natural killer cells. Neutrophil extracellular traps (NETs) are also likely to play a role in binding and destroying microbes.

Hirschfeld *et al.* (2017) reported that *S. gordonii* stimulated neutrophil reactive oxygen species (ROS) release and the bacteria became entrapped within NETs in an *in vitro* study. Recently, evidence has emerged that *S. gordonii* strains with endocarditis pathogenic potential can survive within macrophages at a significantly higher rate than strains without pathogenic potential. Survival within macrophages was due to ROS resistance (Croft *et al.*, 2018).

The complement system (Figure 1.7) is a potent host immune barrier. Complement is triggered after bacterial infection through either the classical, alternative or mannose-binding lectin pathways (Christaki and Giamarellos-Bourboulis, 2014). Opsonins are produced, preparing microbes for phagocytosis and the generation of the membrane attack complex that lyses cells. The host uses regulatory proteins to avoid excessive activation of the complement system.

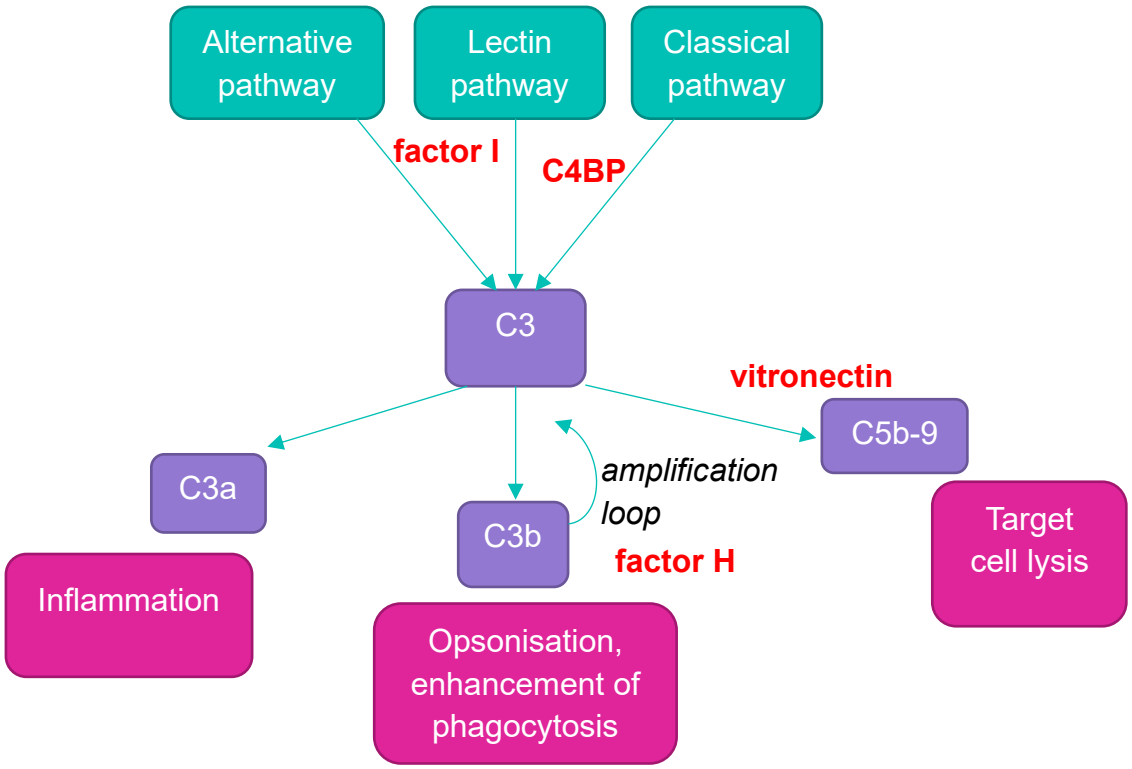

Figure 1.7 Diagrammatic summary of the complement system and its pathways.

The complement system, a humoral component of the innate immune system, comprises approximately 40 plasma and cell membrane-anchored proteins that act in a cascade-like manner to opsonise microbes and facilitate their phagocytosis, initiate inflammation or lyse microbes directly. Negative regulators are depicted in red.

Many microorganisms take advantage of the complement system to enhance virulence (Walport, 2001). Bacteria such as *S. aureus*, *S. pneumoniae* and *S. pyogenes* target these regulatory proteins to escape the host immune response, as well as secreting proteases to degrade components of the complement system (Zipfel, Würzner and Skerka, 2007). One such example is the binding of *S. pneumoniae* PspC to Factor H (FH), a complement control protein and the key soluble negative regulator of C3b amplification, which is a pivotal stage in the complement cascade (Herbert *et al.*,

2015). Vn is another molecule that plays an important role in serum resistance for many Gram-negative bacteria, including *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Moraxella catarrhalis* (Singh, Su and Riesbeck, 2010). Vn is not currently known to play a role in serum resistance for Gram-positive bacteria.

Many of the factors contributing to the ability of oral bacteria to survive in the bloodstream are not fully understood. Roberts *et al.* (2006) reported that post-procedure bacteraemia is quenched within 12 minutes following dental procedures. Reports prior to this study gave estimations varying between less than 5 minutes and more than 180 minutes for bacterial survival in blood (Guntheroth, 1984).

1.6. Association between IE and dental procedures

Although the links between dental procedures and bacteraemia have been known for many decades (Okell and Elliott, 1935), it is still not clear whether dental interventions cause IE (Cahill *et al.*, 2017). The results of a recent large study suggest that invasive dental procedures may be associated with oral streptococcal IE, although the magnitude of this association remains uncertain (Tubiana *et al.*, 2017). Undertaking randomised controlled trials to assess links between dental interventions and IE is challenging due to the rarity of the disease. As a result, a large scale observational study (the IDEA Study) is currently being undertaken linking NHS hospital admissions data to the NHS Business Services Authority Dental Database in the UK (Cahill *et al.*, 2017).

1.6.1. The “dentists are innocent” argument

The relative importance of common, low-level bacteraemia compared with rare, high-magnitude bacteraemia (such as occurs after a dental extraction) remains poorly defined (Cahill *et al.*, 2017). Roberts *et al.* (1999) proposed that everyday transient bacteraemia may be more important than that caused by dental treatment with regards to IE risk. Cumulative bacteraemia levels are thought to be 100,000 times higher during physiological activities, such as flossing or chewing, than after a dental extraction (Que and Moreillon, 2011). The likely importance of physiological bacteraemia is also supported by a recent French study showing that the incidence of oral streptococcal IE in relation to “everyday life” bacteraemia is 94.6 per 100,000 person years among patients with prosthetic heart valves (Tubiana *et al.*, 2017). Only 5% of patients with prosthetic heart valves who developed oral streptococcal IE were recorded as having invasive dental procedures during the 3 months prior to their disease presentation (Tubiana *et al.*, 2017). This is further supported by evidence from animal models, where continuous low-grade bacterial infusion has been reported to be as infective as a single high level bolus in inducing endocarditis (Veloso *et al.*, 2011).

1.6.2. IE related to orthodontics

It is very likely that patients at risk of IE will present for orthodontic treatment. The majority of orthodontic patients present between the ages of 10 and 16, when the incidence of IE is highest in children (Hobson and Clark, 1995). Nevertheless, orthodontically-induced IE is likely to be very rare. Three case reports in total have been published regarding orthodontic treatment-associated IE. Two case reports were

published by Biancaniello and Romero in 1991, describing a 13-year old boy and a 14-year old girl who developed IE caused by *Haemophilus parainfluenzae* and a viridans group *Streptococcus* respectively. Both patients had received an orthodontic adjustment 10 days prior to their disease presentation and neither had undergone any other dental procedures for at least 6 months (Biancaniello and Romero, 1991). In 1993 a further case report was published, describing a 19 year-old with a ventricular septal defect who developed *S. mitis* endocarditis, 10 months after the commencement of orthodontic treatment and 2 weeks after an archwire change and application of powerchain (Hobson and Clark, 1993).

In 1995, the results of a survey of orthodontists were published, reporting that 8 cases of IE associated with orthodontic treatment had occurred over a 44 year period (Hobson and Clark, 1995). Others have estimated that an orthodontist would need to practise for more than 1000 years before being implicated in causing one case of IE (Roberts, Lucas and Omar, 2000). Twelve percent of orthodontists surveyed in the UK had refused orthodontic treatment for at-risk patients. The authors concluded that it was good practice to inform those patients at risk about the possibility of IE arising during orthodontic treatment (Hobson and Clark, 1995). These papers have been useful in showing an association between orthodontic treatment and IE, but causation is difficult to prove, especially in light of the more recent debate regarding bacteraemia arising following physiological activities versus dental treatment. Burden *et al.* (2001) postulated that the relationship between orthodontic treatment and endocarditis was more likely to be coincidental rather than causal. Orthodontic treatment also presents

particular challenges when assessing causation due to the progressive, long-term nature of the treatment in comparison to other dental specialties (Degling, 1972). Nevertheless, the time-course indicated in the case reports described does suggest a relationship.

Studies have attempted to identify which orthodontic procedures are most likely to be linked with bacteraemia and the results have been conflicting (Khurana and Martin, 1999). No bacteraemia was identified following banding and debanding in a study by Degling (1972), but Burden *et al.* (2004) found a bacteraemia prevalence of 13% when brackets and bands were removed. Bacteraemia incidence after orthodontic band placement has been reported as 7.5% and 10% in different studies (McLaughlin *et al.*, 1996; Erverdi *et al.*, 2000). It has also been highlighted that even when no obvious bleeding was detected clinically, post-operative bacteraemia can be detected (McLaughlin *et al.*, 1996). Further investigations have been carried out to assess incidence of bacteraemia following mini-implant insertion (1 in 40, Uysal *et al.,* 2010), mini-implant removal (no bacteraemia, Livas *et al.,* 2013), orthodontic stripping (1 in 29, Yagci *et al.,* 2013), gold chain adjustment (no bacteraemia, Lucas *et al.,* 2007), piezocision (1 in 10, Ileri *et al.,* 2014) and removal of Haas palatal expanders (1 in 2, Rosa *et al.,* 2005). Many of these studies provide conflicting evidence and often the sample sizes are small. There may also be limitations associated with the methodology in these papers. Broth culture is often used, allowing the percentage prevalence of positive blood culture to be recorded. However, this technique is unable to provide information regarding the number of bacteria in the blood sample that was originally

taken. The lysis filtration method can provide both an estimate of the prevalence as well as the intensity of the bacteraemia.

The lysis filtration technique was used by Lucas *et al.* (2002) to identify bacteraemia prevalence and intensity after a number of orthodontic procedures, including alginate impressions, separator placement, band placement and fixed appliance archwire adjustment. Separator placement was the only intervention that resulted in a significant bacteraemia. A further study confirmed that bacteraemia occurred following separator placement and that viridans group *Streptococcus* bacteria were the predominant isolates, alongside *S. aureus* (Umeh *et al.*, 2016). Lucas *et al.* (2002) reported on the bacteria most commonly found in blood cultures following orthodontic procedures, with 65% being coagulase-negative staphylococci and 9% being oral streptococci, with *Streptococcus vestibularis, S. mitis*, *S. salivarius, S. gordonii* and *S. sanguinis* making up this group. *S. sanguinis* has also been detected following insertion of mini-implants and orthodontic stripping (Uysal *et al.*, 2010; Yagci *et al.*, 2013). Piezocision and removal of Haas palatal expanders have both been associated with *S. oralis* bacteraemia (Rosa *et al.*, 2005; Ileri *et al.*, 2014).

Orthodontic treatment-related bacterial colonisation of a non-cardiac site has also been reported. A brain abscess due to *Haemophilus aphrophilus* (a Gram-negative bacillus and a component of dental plaque) occurred in a 12 year-old boy. He had started fixed appliance therapy 6 weeks prior and had received a fixed appliance adjustment two weeks prior to disease presentation. The authors of the case report

postulated that this was due to transient bacteraemia as a result of the orthodontic appliance manipulation and that a patent foramen ovale had allowed the bacteria to enter the cerebral circulation (Wolf and Curtis, 2008).

1.6.3. Clinical guidelines regarding dentistry and bacteraemia risk

The clinical management of patients at risk of IE undergoing dental procedures has been a controversial topic for several years. NICE issued guidelines in 2008 withdrawing the prescription of antibiotic prophylaxis for patients at risk of IE undergoing dental procedures. A large longitudinal study showed an increase in incidence of IE cases after this change in practice (Dayer *et al.*, 2015). However, limitations of this study included the fact that data were obtained from hospital coding and that it was not possible to distinguish between the effects of subtle changes in the population due to immigration or other health policy from the overall trend. It should also be stressed that the association between IE incidence and reduced antibiotic prescriptions was not necessarily causal. Nevertheless the results of this study were a trend mirrored by increased incidence of IE in some studies from the USA and Germany. These rises in incidence had occurred after implementation of American and European guidelines (2007 and 2009/2015 respectively), recommending antibiotic prophylaxis only be given in patients with prosthetic heart valves, a history of IE or with a congenital cyanotic heart disease undergoing invasive dental procedures (Wilson *et al.*, 2007; Habib *et al.*, 2009; Habib *et al.*, 2015).

NICE reviewed their guidelines in 2015 and reported that there was insufficient evidence to warrant a change, stimulating considerable debate in the literature (Alderson and Baker, 2016; Chambers *et al.*, 2016a; Chambers *et al.*, 2016b; Million *et al.*, 2016; Thornhill *et al.*, 2016c; Thornhill *et al.*, 2016d). In July 2016, there was a change in the wording of the NICE guidelines, meaning that in individual cases, antibiotic prophylaxis may be appropriate (NICE 2008, updated 2016). This leaves considerable challenges for the dental profession in implementation of the guidelines (Thornhill *et al.*, 2016a), but recent publication of "Implementation Advice for National Institute for Health and Care Excellence (NICE) Clinical Guideline 64 *Prophylaxis Against Infective Endocarditis"* by the Scottish Dental Clinical Effectiveness Programme has been welcomed. It is the orthodontist's responsibility to liaise carefully with patients' physicians and other healthcare professionals to act in the patient's best interests, whilst adhering to the principles of informed consent, as described in the Montgomery case in 2015 (Main and Adair, 2015).

1.6.4. Clinical guidelines regarding orthodontics and bacteraemia risk

Good guidelines for the management of orthodontic patients at risk of IE have been lacking or conflicting (Khurana and Martin, 1999). The current discrepancies in guidelines for antibiotic prophylaxis for patients undergoing dental procedures across different parts of the world complicate the picture. Although performed before the change in NICE guidelines, a study in the field of IE risk management in the UK found that compliance is often compromised when complex, conflicting guidelines exist. The need for simple, unambiguous guidelines was highlighted (Chate, 2008). In America,

there is confusion about which orthodontic procedures are most prone to generate bacteraemia amongst orthodontists (Leong, Kunzel and Cangialosi, 2012).

In addition to identifying patients who may benefit from antibiotic prophylaxis, the use of antimicrobial mouth rinses has also been proposed for some orthodontic patients. Chlorhexidine gluconate-based mouth rinses may be beneficial in temporarily reducing bacterial load prior to placement or removal of bands (Sonis, 2004). A more recent study by Dua *et al.* (2016) found that the use of a chlorhexidine rinse immediately prior to separator placement resulted in no bacteraemia being detected post-procedurally. However, this study was flawed due to the lack of a control group.

Orthodontists have a major role to play in surveillance and education. They may be the only health care professionals that fit and healthy young individuals see on a regular basis. The orthodontic profession therefore has a duty of care in education, prevention and early detection of IE. The following suggestions have been made for orthodontists treating patients at risk of IE (Patel, Burden and Sandler, 2009):

- The orthodontic team should stress the importance of good general dental and skin hygiene
- Communication with the patient's physician enables the orthodontist to understand the risk of IE for each susceptible patient
- Orthodontists should provide information about the signs and symptoms that may indicate IE and when to seek specialist advice

- The use of orthodontic bands and fixed acrylic appliances should be avoided in high risk patients with poor oral hygiene
- There should be prompt referral, investigation and treatment of any episode of infection

These principles are mirrored in the British Orthodontic Society's guidelines for the management of patients at risk of IE (BOS, 2016), where it is stipulated that if in doubt, the orthodontist should always contact the patient's cardiac specialist.

1.7. Summary and implications for further research

Although rare, infection-induced cardiovascular disease has high mortality and morbidity rates and places a large burden on society. There is a lack of understanding regarding the mechanisms underlying survival and virulence of oral streptococci in the bloodstream, both critical prerequisites for streptococcal IE. Oral streptococcal blood survival mechanisms may represent putative targets for the prevention or treatment of IE. To develop this strategy, a detailed understanding of the molecular basis of survival of oral streptococci in blood is required. Critical questions are:

- How long do oral streptococci survive in the blood?
- Do oral streptococci modulate the host complement system to survive in blood?
- What bacterial factors are important in evasion of host defences?
- Are streptococcal survival mechanisms likely to be applicable across other endocarditis pathogens?

- What are the implications of these findings for the management of orthodontic and paediatric patients at risk of IE in the context of the current regimes in the UK?

1.8. Aims and Objectives

The **overall aim** of this project was to study the blood survival mechanisms of microorganisms commonly implicated in orthodontic separator placement, with a particular focus on oral streptococci. This project will therefore help to develop a mechanistic understanding of streptococcal virulence in bacteraemia and IE.

The **specific objectives** were to:

- Identify, from published literature, relevant oral streptococci for use in blood survival studies
- Determine the time course of survival for different oral streptococci in blood
- Investigate the role of bacterial surface determinants in promoting streptococcal survival in blood
- Characterise interactions of oral streptococci with complement factors relevant for host immune evasion

2. Materials and Methods

2.1. Growth conditions

Streptococcal strains were grown in Brain Heart Infusion broth supplemented with 0.5% Yeast Extract (BHY) at 37 °C in reduced oxygen conditions. Lactococcal strains were grown in Brain Heart Infusion broth supplemented with 0.5% Yeast Extract (BHY) at 30 °C in reduced oxygen conditions. Where required, media were supplemented with antibiotics at the following concentrations: erythromycin, 5 μg/ml; spectinomycin, 100 μg/ml. Bacterial strains used in this project are listed in Table 2.1.

Table 2.1 Bacterial strains used in this project

Organism	Strain	Relevant genotype	University of Bristol (UB) number	Source or Reference
S. gordonii	DL1	Wild-type, parental strain Challis	1507	Le Blanc, Lee and Inamine, 1991
S. mitis	NCTC 12261	Wild-type	398	Kilian, Mikkelsen and Henrichsen, 1989
S. salivarius	NCTC 8606	Wild-type	384	Willers, Ottens and Michel, 1964
S. oralis	NCTC 11427	Wild-type	2178	Ronda, García and López, 1988
S. oralis	CR 834	Wild-type	2627	Oral cavity (Douglas, Brown and Preston, 1990)
S. sanguinis	SK36	Wild-type	1330	Mogens Kilian, first genome-sequenced strain (Kilian, Mikkelsen and Henrichsen, 1989)
S. sanguinis	2017-78	Wild-type	612	Confirmed case of subacute bacterial endocarditis (Herzberg, Brintzenhofe and Clawson, 1983)
S. gordonii	DL1	Δ*padC*	2889	Laboratory strain
S. gordonii	DL1	Δ*padA*	2864	Laboratory strain

S. gordonii	DL1	Δ*padB*	2833	Laboratory strain
S. gordonii	DL1	Δ*hsa*	2930	Laboratory strain
S. gordonii	DL1	Δs*ndA*	2827	Laboratory strain
S. gordonii	DL1	Δ*sortase A*	2576	Nobbs *et al.,* 2007b
S. gordonii	DL1	*Δhsa – pMSP-hsa*	2937	Laboratory strain
S. gordonii	DL1	*ΔpadA::aad9*	2723	Haworth *et al.*, 2017a
S. gordonii	DL1	*ΔpadA::aad9-pMSP-padA*	2724	Haworth *et al.*, 2017a
S. gordonii	DL1	Δ*padA*-pMSP–*padA*-His-term	2870	Laboratory strain
S. gordonii	DL1	Δ*hsa*-pMSP-*hsa*-His-term*(+)*	2935	Laboratory strain
Lactococcus lactis	MG1363	Wild-type Lac^-	754	Gasson, 1983

2.2. Whole blood preparation

Informed consent was obtained and whole blood was collected from the antecubital fossa of healthy volunteers who were either staff or students of the University of Bristol. In total, 11 volunteers donated blood for this project. Varying numbers of donors were used for each individual experiment. The ethical approval is described in 2.12 below. Exclusion criteria included pregnancy, medical treatment, current medication, being below the age of 18 and feeling unwell. The blood was collected into 10 ml plastic vacutainer bottles containing K_2EDTA (Fisher Scientific, Loughborough, UK) and inverted 10 times to ensure thorough mixing with the anticoagulant.

2.3. Bacterial blood or serum survival assay

Bacterial cultures (10 ml) were grown overnight at 37 °C in a candle jar and cells harvested by centrifugation (5000 x *g*, 7 min) using a Rotina 380 benchtop centrifuge (Hettich, Massachusetts, USA). Cells were washed once in PBS (phosphate buffered saline) and adjusted in PBS to OD_{600} 1.0 (equivalent to $1x10^9$ cells/ml). Anticoagulated human blood (90 µl) or human serum (90 µl, CS500-100, TCS Biosciences, Buckingham, UK) was added to the wells of a 96-well plate. PBS or adjusted cell suspension (10 µl) was added to the appropriate wells and mixed. Microtitre plates were incubated in a candle jar at 37 °C for 0, 2, 4 or 6 h at 80 rpm. The bacteria/blood or bacteria/serum suspensions were serially diluted in PBS and aliquots (10 µl) plated onto agar plates. The plates were incubated for 24 h at 37 °C in a candle jar and the colony forming units counted.

2.4. Immunodot blot analysis

Bacterial suspension (2 µl), adjusted to OD_{600} 1.0 in PBS, was placed onto nitrocellulose and the membrane allowed to air dry. The blot was blocked in 5% BSA in TBST (Tris-buffered saline [TBS; 10 mM Tris-HCl pH 7.5, 100 mM NaCl] supplemented with 0.1% Tween-20) overnight at 4 °C. The membrane was washed twice in TBST (50 ml, 10 min). FH was detected by probing the nitrocellulose membrane with mouse anti-human FH antibody (1 in 1000 dilution, Biorad, Watford, Hertfordshire) in TBST (45 min) followed by washing in TBST (15 min) and two further washes in TBST (5 min). The membrane was probed with goat anti-mouse horseradish peroxidase-conjugated antibody (1 in 2000 dilution, Dako, Santa Clara, USA) in TBST (45 min) followed by washing in TBST (15 min) then four further

washes in TBST (5 min). The blot was incubated with enhanced chemiluminescence Western blot detection reagent (GE Healthcare, Amersham, Buckinghamshire, UK) and exposed to film in the dark and subsequently developed. Densitometry analysis of immunodot blots was performed using Image Quant TL software (GE Healthcare, Amersham, Buckinghamshire, UK).

2.5. Bacterial binding assay to FH in human serum

Bacterial cultures (10 ml) were grown overnight at 37 °C in a candle jar and cells harvested by centrifugation (5000 x *g*, 7 min). Cells were washed once in PBS^+ (PBS supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and adjusted to OD_{600} 1.0. The bacterial suspension (1 ml) was centrifuged (5000 x *g*, 7 min) and the cell pellet resuspended in 10% human serum (TCS Biosciences CS500-100; 1 ml; diluted in PBS^+) and incubated with gentle agitation at 37 °C (30 min). The bacterial suspension was incubated on ice (1 min) to inhibit complement activation. Bacterial cells were harvested by centrifugation (13000 *g*, 4 °C, 5 min), washed once in PBS^+ then resuspended in PBS containing 0.05% (v/v) sodium azide for analysis by immunodot blot assay. FH binding was detected by probing with primary antibody (mouse anti-human FH, 1 in 1000 dilution, Biorad) and secondary antibody (goat anti-mouse horseradish peroxidase-conjugated, 1 in 2000 dilution, Dako). Reactivity was detected as described for immunodot blot analysis.

2.6. Crystal violet bacterial binding assay to human FH

Human FH (50 µl, 20 µg/ml final concentration, Merck, Darmstadt, Germany), diluted in coating buffer (20 mM Na_2CO_3, 2 mM $NaHCO_3$, pH 9.3), was added to wells of a high-binding 96-well plate (Immulon 2HB, Thermo Scientific) and

incubated overnight at 4 °C. Wells were washed once in 200 µl TBSC (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 5 mM $CaCl_2$), and non-specific binding sites blocked with 3% (w/v) BSA in TBSC containing 0.05% (v/v) Tween-20 for 1 h at 37 °C. Bacterial cultures (10 ml) were grown overnight at 37 °C (streptococci) or 30 °C (lactococci) in a candle jar and cells were harvested by centrifugation (5000 x *g*, 7 min). Bacterial cells were washed once in TBSC (5 ml) and the suspension adjusted in TBSC to OD_{600} 1.0. The adjusted cell suspension (100 µl) was added to the wells and incubated at 37 °C for 2 h. Following removal of the suspension, the wells were washed twice in TBS (200 µl). Adherent cells were fixed by adding 25% (v/v) formaldehyde (100 µl) for 30 min at room temperature. Wells were washed twice in TBS (200 µl) before adding 0.5% (w/v) crystal violet (100 µl) and incubating for 2 min at room temperature. Wells were washed three times in TBS before adding 10% (v/v) acetic acid (100 µl) and incubating at room temperature for 5 min. Levels of adhesion were quantified by measuring absorbance at 595 nm.

2.7. Binding of bacteria to pure FH

Bacterial cultures (10 ml) were grown overnight at 37 °C (streptococci) or 30 °C (lactococci) in a candle jar and cells were harvested by centrifugation (5000 x *g*, 7 min). Bacterial cells were washed once in PBS^+ and the suspension adjusted in PBS^+ to OD_{600} 1.0. Bacterial cells were harvested by centrifugation (13800 x *g*, 5 min), resuspended in human FH (500 µl, diluted in PBS^+ to 50 µg/ml, Merck) and incubated with gentle agitation (1 h, 37 °C). Cells were washed twice in PBS^+ (5 ml), resuspended in 0.2 M glycine-HCl (2.5 ml, pH 2.6) and incubated at room temperature with gentle agitation (15 min). The bacterial suspension was

centrifuged (13800 x *g*, 5 min), the supernatant collected, and the pH adjusted to pH 7 by the addition of Tris-HCl (1 M, pH 8.0, 1 ml). The solution was concentrated 10-fold in a refrigerated Centrivap concenthumarator (LabConco, Kansas City, USA), and the concentrated solution (2 µl) placed onto a nitrocellulose membrane and allowed to air dry. The presence of FH was detected by immunodot blot analysis, as described above (2.4).

2.8. Recombinant protein FH binding ELISA

Human FH (50 µl, 20 µg/ml, Merck), diluted in coating buffer, was added to wells of a high-binding 96-well plate (Immulon 2HB) and incubated overnight at 4 °C. Wells were washed once in 200 µl TBSC and non-specific binding sites blocked with 3% (w/v) BSA in TBSC (200 µl) for 1 h at 37 °C. Wells were washed once in TBSC (200 µl) before recombinant protein (1, 2 or 5 µg, 50 µl final volume), diluted in TBSC, was applied to the wells and incubated for 1 h at 37 ^{o}C. Unbound protein was removed and wells washed once in TBS (200 µl). Primary antibody (mouse anti-tetraHis, 50 µl, Qiagen, Manchester, UK), diluted 1 in 1000 into TBST, was added to the wells and incubated for 1 h at 37 ^{o}C. Wells were washed twice in TBST (200 µl) before adding secondary antibody (goat anti-mouse HRP, diluted 1 in 2000 into TBST containing 3% [w/v] BSA, 50 µl, Dako) and incubating for 1 h at 37 ^{o}C. Wells were washed once in TBST (200 µl) and then twice in TBS (200 µl). ELISA reagent (0.102 M Na_2HPO_4, 0.049 M citric acid, 0.012% H_2O_2, 3.7 mM *O*-phenylenediamine; 50 µl) was added to wells and incubated in the dark for 10 min at room temperature. H_2SO_4 (0.65 M, 50 µl) was added to stop the reaction. Levels of adhesion were quantified by measuring absorbance at 490 nm.

2.9. ELISA to detect binding of FH to native $PadA_{His6}$ or Hsa_{His6} on Ni-NTA HisSorb strips

Ni-NTA HisSorb strip wells (Qiagen) were coated with native $PadA_{His6}$ (200 µl, 100 µg/ml) or Hsa_{His6} (200 µl, 100 µg/ml) in TBS containing 3% (w/v) BSA and incubated at room temperature for 1 h. Wells were washed four times in TBSC (200 µl) and FH (200 µl, 5 µg/ml, Merck), diluted in TBST containing 3% (w/v) BSA, was added to wells and incubated at room temperature (37 °C, 1 h). The remainder of the assay was performed as described in section 2.8 above. All incubation steps were performed at 90 rpm.

2.10. Crystal violet bacterial binding assay to human Vn

Multimeric Vn (5 µg/ml final concentration, Merck) was diluted in coating buffer (20 mM Na_2CO_3, 2 mM $NaHCO_3$, pH 9.3) and incubated with mAb 8E6 (10 µg/ml, Sigma, Darmstadt, Germany) for 1 h (37 °C, 50 rpm). Multimeric Vn (5 µg/ml final concentration, Merck), diluted in coating buffer, or multimeric Vn that had been pre-incubated with mAb 8E6, was added to wells of a high-binding 96-well plate (Immulon 2HB) and incubated overnight at 4 °C. Wells were washed once in 200 µl TBSC and non-specific binding sites blocked with 3% (w/v) BSA in TBSC containing 0.05%(v/v) Tween-20 for 1 h at 37 °C. Bacterial cultures (10 ml) were grown overnight at 37 °C (streptococci) or 30 °C (lactococci) in a candle jar and cells were harvested by centrifugation (5000 x *g*, 7 min). The crystal violet binding assay was performed as described in 2.6.

2.11. ELISA to detect binding of Vn to immobilised *S. gordonii* DL1 WT cells

Multimeric Vn (5 µg/ml final concentration, Merck), diluted in TBSC, was incubated with heparin (final concentration 180 µg/ml, Sigma) for 1 h (37 ^{o}C, 50 rpm). *S. gordonii* DL1 WT bacterial cells from overnight culture were washed, resuspended to OD_{600} 1.0 and immobilised in microtitre plate wells. Wells were washed once in 200 µl TBSC and non-specific binding sites blocked with 3% (w/v) BSA in TBSC (200 µl) for 1 h at 37 ^{o}C. Wells were washed once in TBSC (200 µl) before multimeric Vn (5 µg/ml final concentration, Merck), diluted in TBSC, or multimeric Vn that had been pre-incubated with heparin (5 µg/ml final concentration), was applied to the wells and incubated for 1 h at 37 ^{o}C. Unbound protein was removed and wells washed once in TBS (200 µl). Primary antibody (50 µl, mouse anti-human Vn, Santa Cruz Biotechnology, Texas, USA), diluted 1 in 1000 into TBST, was added to the wells and incubated for 1 h at 37 ^{o}C. Wells were washed twice in TBST (200 µl) before adding secondary antibody (goat anti-mouse HRP, diluted 1 in 2000 into TBST containing 3% [w/v] BSA, 50 µl, Dako) and incubating for 1 h at 37 ^{o}C. The remainder of the assay was performed as described in 2.8.

2.12. Ethical approval

Ethical approval was gained from the University of Bristol Health Sciences Faculty Research Ethics Committee (application number 54322) for obtaining blood from University of Bristol staff and student volunteers under the study title "Understanding the link between oral microbes and systemic disease".

2.13. Statistical analysis

Statistical analyses were performed using SPSS (IBM) or Excel (Microsoft Office 365 Plus). Data shown are the mean ± standard error of the mean (SEM). Unless otherwise stated, experiments were performed in triplicate. Comparisons between the means were performed using the Student's 2-tailed unpaired *t* test or, where multiple samples were compared, by ANOVA followed by the Tukey test. Individual data points are shown for blood survival studies in scatter plots and different blood donors are depicted by different colours. Comparisons between strains for the blood survival data were performed using the Wilcoxon signed ranks test or, where multiple samples were compared, by the Friedman test. For all data shown, a *P* value of <0.05 was considered significant.

3. Results

3.1. Survival of bacteria in human blood

The ability of bacteria to survive in the bloodstream is likely to be critical for IE pathogenesis. Estimations of duration for bacterial survival in blood have varied between less than 5 minutes to more than 180 minutes (Guntheroth, 1984; Roberts *et al.*, 2006), and it is possible that IE disease risk may be directly related to the length of time bacteria can survive. Initial studies therefore aimed to investigate the time course of bacterial survival in human whole blood. Oral viridans group *S. gordonii* is an important cause of IE and was used as a reference species in these initial investigations. This microorganism colonises most surfaces in the oral cavity and its virulence mechanisms in IE have been widely investigated (Keane *et al.*, 2010; Petersen *et al.*, 2010; Haworth *et al.*, 2017a).

Fresh human whole blood was selected for use in these *in vitro* studies to provide the most relevant experimental conditions possible. Initially, the ability of *S. gordonii* to survive in human blood was compared to that of *L. lactis*. *L. lactis* is a Gram-positive bacterium commonly used as a heterologous expression host for Gram-positive surface proteins. *L. lactis* does not colonise human tissues naturally and is a 'generally regarded as safe' (GRAS) species, making it a suitable negative control. Following 2 h incubation in human blood, bacteria were recovered and the numbers of viable cells determined by measurement of colony-forming units grown on agar plates following an overnight incubation (Figure 3.1).

Figure 3.1 Appearance of colony-forming units on agar plate, divided into 6 sections according to serial dilution. See Methods 2.3. The plates were incubated for 24 h at 37 ℃ in a candle jar and the colony-forming units counted.

Survival at 2 h is presented as a percentage of the initial inoculum in Figure 3.2.

After 2 h, *L. lactis* was reduced in survival compared to *S. gordonii* for both donors, although the difference was not statistically significant (Figure 3.2).

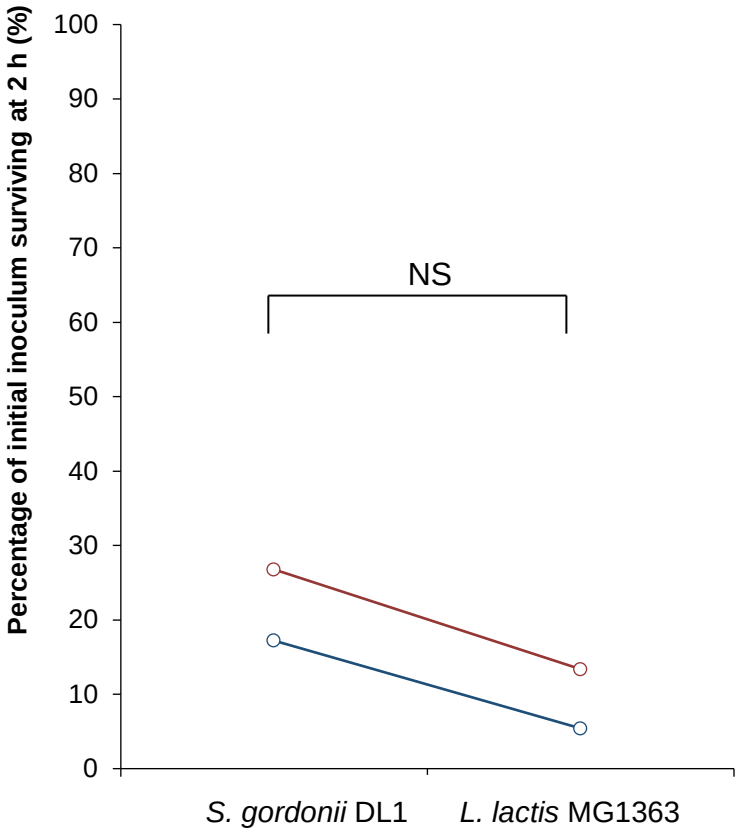

Figure 3.2 Percentage survival of wild-type *S. gordonii* and *L. lactis* bacterial cells incubated in human whole blood for 2 h. See Method 2.3. Each coloured line connecting data points indicates data from individual donors. *P*=NS as determined by Wilcoxon signed rank test; n=2.

S. gordonii was further investigated for ability to survive over a 6 h time course in human blood (Figure 3.3). Percentage survival reduced between 2 h and 4 h for all the donors and continued to reduce between 4 h and 6 h for nearly half the donors.

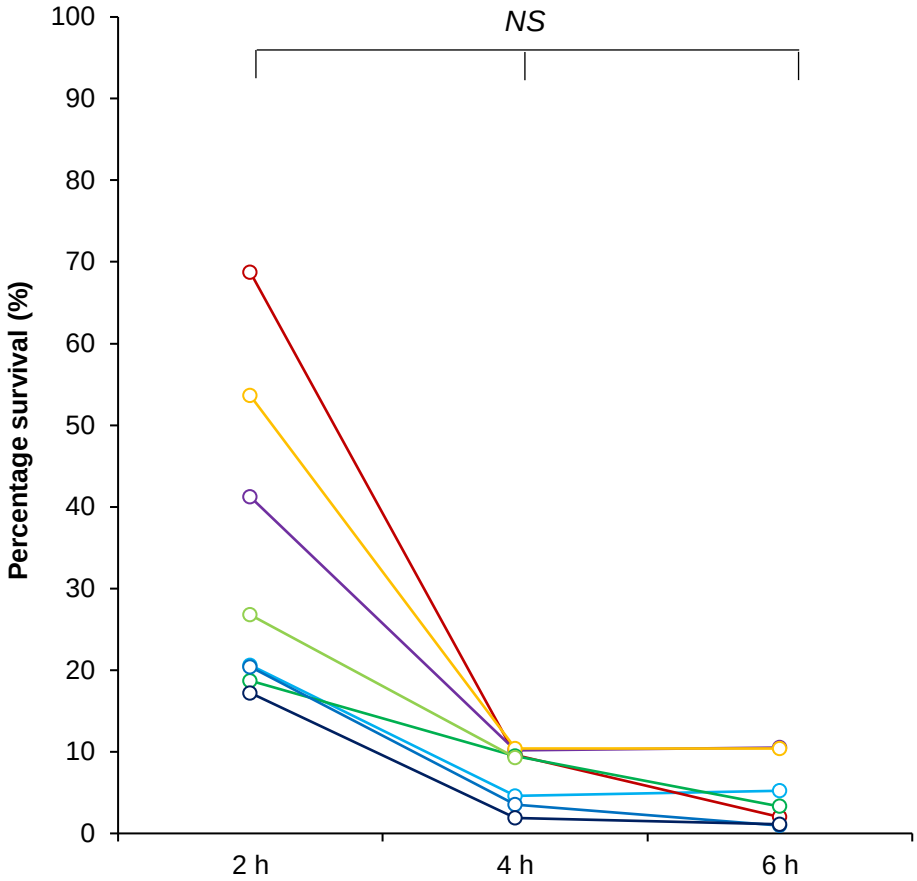

Figure 3.3 Percentage survival of wild-type *S. gordonii* incubated in human whole blood over 6 h. See Method 2.3. Each coloured line connecting data points indicates data from individual donors. *P*=NS as determined by Friedman test and post-hoc analysis with Wilcoxon signed-rank tests (with Bonferroni correction applied). n=7.

Other streptococcal endocarditis pathogens were selected for comparison with *S. gordonii*. Gram stain micrographs of the strains selected are shown in Figure 3.4 and confirmed purity of all the strains.

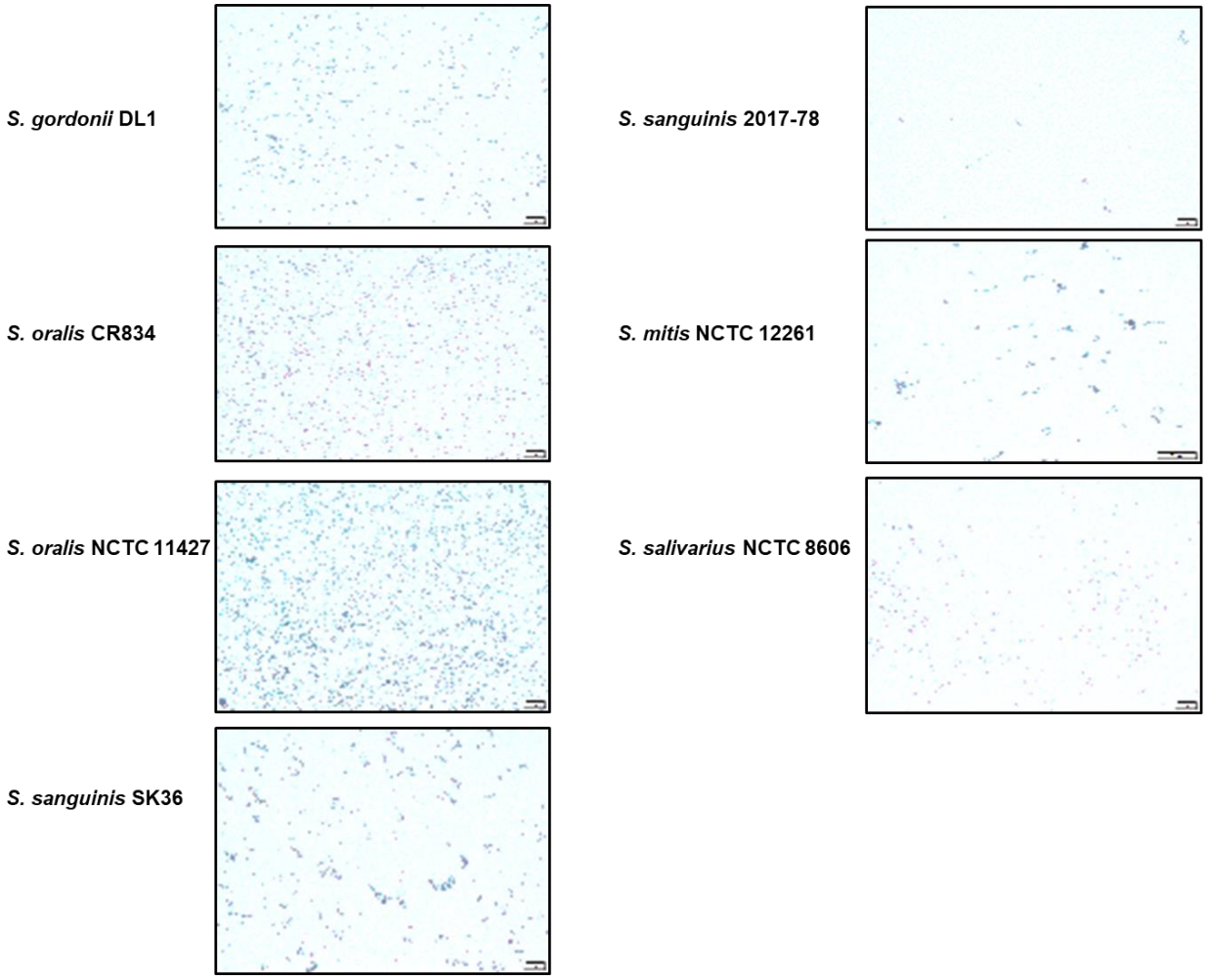

Figure 3.4 Representative Gram stains of streptococcal strains used in this project, showing no evidence of sample contamination

IE pathogen *S. oralis* was selected as a second member of the mitis group of streptococci, alongside *S. gordonii*. After 2 h incubation, *S. oralis* CR843 showed slightly lower or similar survival percentages and *S. oralis* NCTC 11427 increased survival percentages compared to *S. gordonii* DL1 for 3 out of the 4 donors. One donor showed increased survival for *S. oralis* CR843 and decreased survival for *S. oralis* NCTC 11427 compared to *S. gordonii* DL1 (Figure 3.5). Overall, the differences between the strains were not statistically significant.

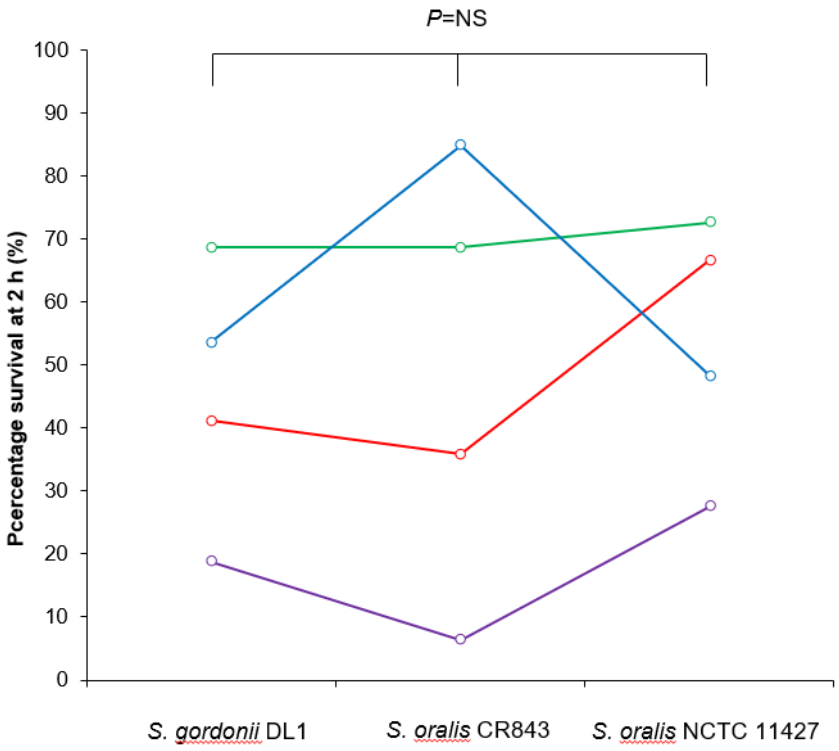

Figure 3.5 Percentage survival of *S. gordonii* and *S. oralis* strains incubated in human whole blood for 2 h. Method 2.3. Each coloured line connecting data points indicates data from individual donors. *P*=NS (not significant) as determined by Friedman test; n=4.

S. sanguinis is closely related to *S. gordonii* and, while often isolated in abundance from health-associated microbial communities (Nobbs and Kreth, 2019), has the potential to cause IE (Do *et al.*, 2011). Strains *S. sanguinis* 1330 and 2017-78 were chosen for investigation, with the latter being isolated from a confirmed case of subacute bacterial endocarditis (Herzberg, Brintzenhofe and Clawson, 1983). Two out of 3 donors showed increased survival for *S. sanguinis* SK36 compared to *S. gordonii* DL1. *S. sanguinis* 2017.78 survived at levels either slightly greater or slightly lower than that of *S. gordonii* DL1 (Figure 3.6).

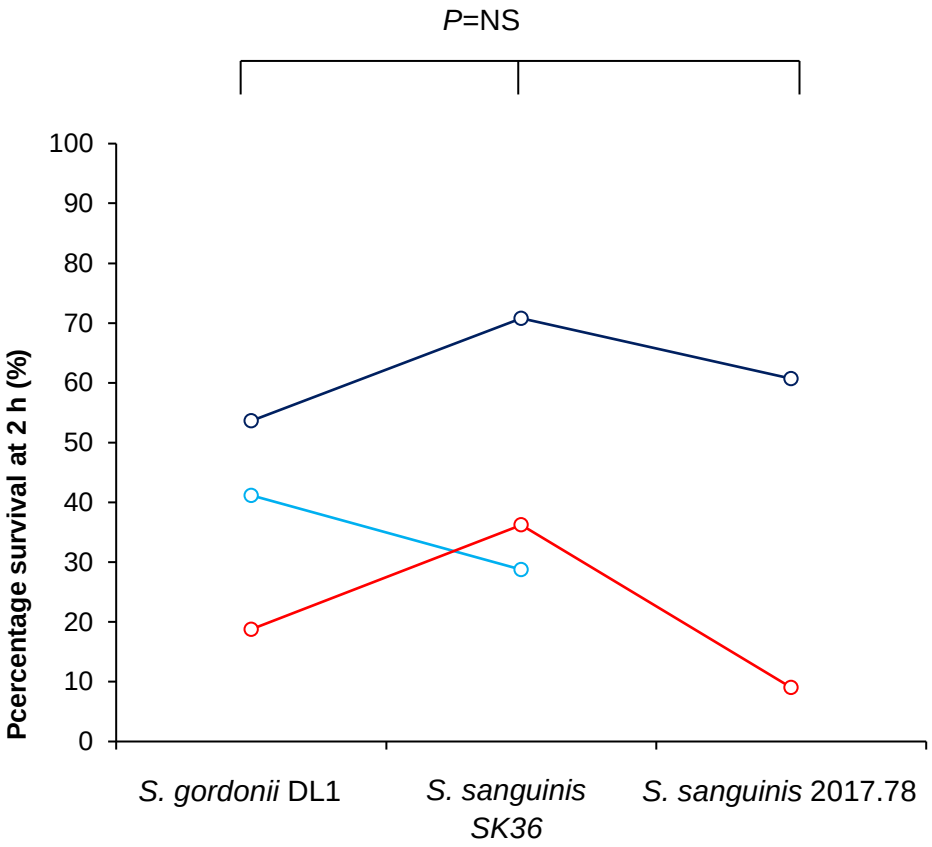

Figure 3.6. Percentage survival of *S. gordonii* and *S. sanguinis* incubated in human whole blood for 2 h. Method 2.3. Each coloured line connecting data points indicates data from individual donors. *P*=NS (not significant) as determined by Friedman test; n=3 (*S. sanguinis* 2017.78, n=2, as bacteria failed to grow for one experiment)

S. salivarius is a member of the salivarius group of streptococci and has no strong association with cardiovascular disease. *S. salivarius* NCTC 8606 showed reduced survival levels compared to *S. gordonii* DL1 for all donors. *S. mitis* NCTC 12261, a third member of the mitis group of streptococci and a known causative agent of IE was also reduced in survival compared to *S. gordonii* DL1 at 2 h (Figure 3.7).

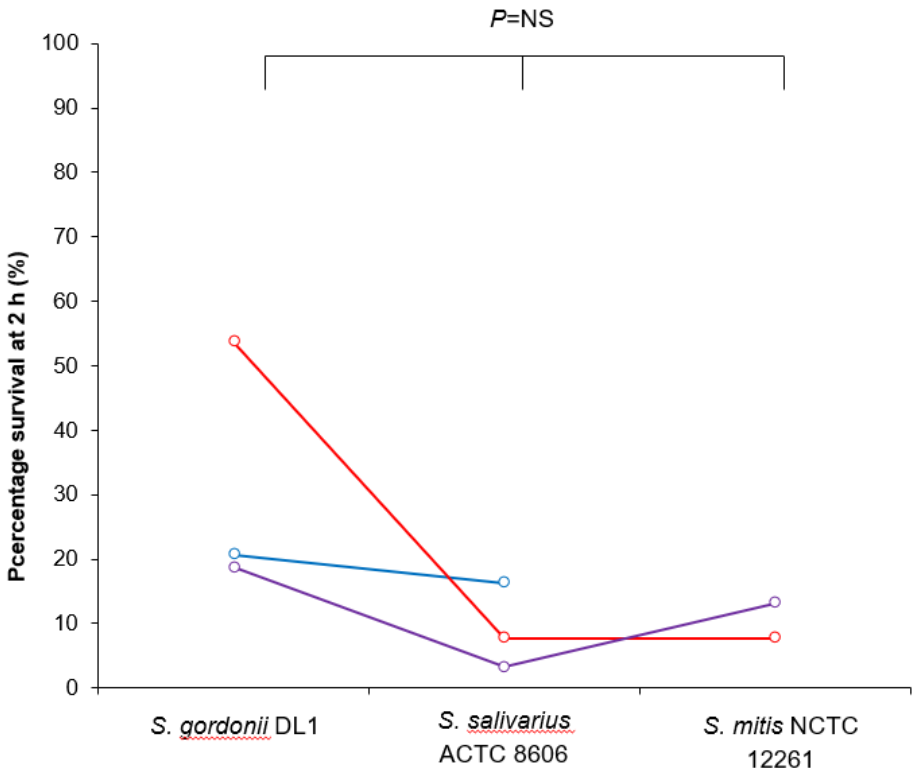

Figure 3.7 Percentage survival of *S. gordonii*, *S. mitis* and *S. salivarius* incubated in human whole blood for 2 h. Method 2.3. Each coloured line connecting data points indicates data from individual donors. *P*=NS (not significant) as determined by Kruskal Wallis test; n=3 (*S. mitis* NCTC 12261, n=2, as bacteria failed to grow for one experiment).

Taken together, these data showed that survival in human blood by oral *Streptococcus* bacteria was species- and strain-dependent, with those associated with IE demonstrating a general trend of greater capacity to withstand clearance. Of the VGS that were tested, survival levels were all comparable or lower than those of *S. gordonii*. This validated the use of *S. gordonii* as the model strain for exploring in greater detail the mechanistic basis for survival in blood.

3.2. Interaction with complement cascade mediators

The complement system, an important component of the innate immune response, comprises many plasma proteins that act in a cascade-like manner. To prevent the host's own activated complement causing unintended injury to self cells, it is critical that host cells have a number of proteins capable of down-regulating the complement cascade. A set of plasma proteins including FH, Vn, factor I, C4BP and C1 have a pivotal role in preventing complement activation in host tissues.

3.2.1. Interaction with FH

FH is a conserved plasma glycoprotein that inhibits the alternative complement pathway and the C3b amplification loop. It has a molecular weight of ~150 kDa, and is synthesised by hepatocytes and secreted into the blood at concentrations up to 500 µg/ml. The FH molecule comprises 20 complement control protein domains, with the regulatory activities of FH being mediated by N-terminal domains 1-4, which include a C3b-binding site (Jozsi, 2017). The C-terminal domains anchor the molecule to host surfaces with deposited C3b (Jozsi, 2017), enabling FH to protect the cells from complement-mediated damage. Several microbes are known to exploit this mechanism and recruit FH to the microbial surface to escape

complement-mediated killing, including *S. pneumoniae* (Janulczyk *et al.*, 2000) and *N. meningitidis* (Pizza, Donnelly and Rappuoli, 2008). Unpublished mass spectrometry data (Lohiya, 2018) had suggested that two large cell surface-anchored proteins of *S. gordonii*, PadA and Hsa, were able to interact with FH from human serum. Possible binding of PadA or Hsa to FH was therefore investigated further as a putative mechanism for enhancing *S. gordonii* survival in blood.

Initially, Hsa and PadA proteins, purified from *S. gordonii*, were investigated for ability to bind FH directly. *S. gordonii* strains UB2870 Δ*padA*/pMSP-*padA*-His-term and *S. gordonii* UB2935 Δ*hsa/*pMSP-*hsa*-His-term, which secrete native forms of PadA or Hsa, respectively, carrying a C-terminal His6 tag ($PadA_{His6}$ or Hsa_{His6}), had been previously generated. These strains were cultured in FMC medium supplemented with nisin (100 ng/ml) to induce expression and secretion of $PadA_{His6}$ or Hsa_{His6} into the culture medium. This was subsequently recovered and the protein purified using nickel affinity chromatography. To investigate binding of $PadA_{His6}$ or Hsa_{His6} to purified FH, an ELISA assay was performed. $PadA_{His6}$ or Hsa_{His6} were immobilised onto Ni^{2+}-coated microtitre wells, before detecting purified FH bound from solution with anti-FH antibody. These assays showed significant binding by FH to $PadA_{His6}$ and Hsa_{His6} when compared to the buffer only control (Figures 3.8 and 3.9).

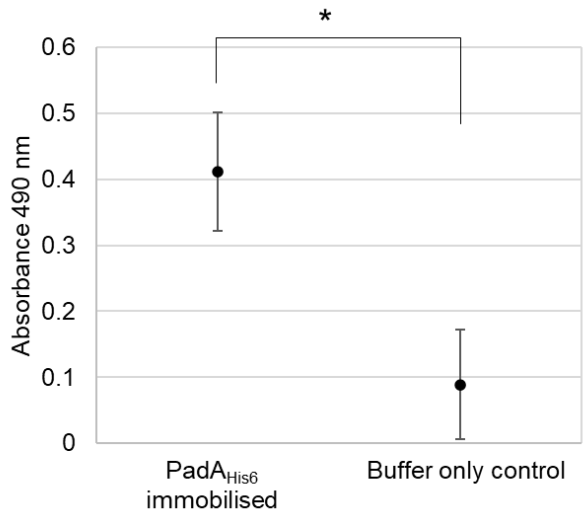

Figure 3.8 FH adhesion to immobilised PadA$_{His6}$ was determined by ELISA. FH binding was detected by mouse anti-FH antibody (1:1000 dilution) and secondary anti-mouse HRP antibody (1:2000 dilution). ELISA reagent was added and absorbance read at 490 nm. Error bars indicate SD. *P=0.01 as determined by Student's t test; n=3.

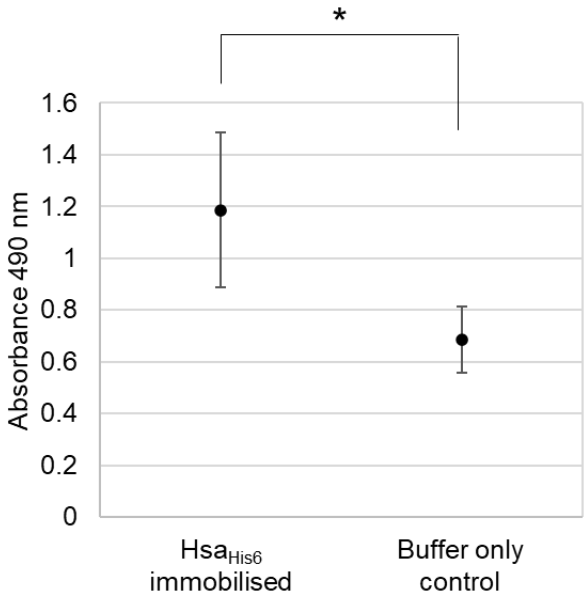

Figure 3.9 FH adhesion to immobilised Hsa$_{His6}$ was determined by ELISA. FH binding was detected by mouse anti-FH antibody (1:1000 dilution) and secondary anti-mouse HRP antibody (1:2000 dilution). ELISA reagent was added and absorbance read at 490 nm. Error bars indicate SD. *P<0.01 as determined by Student's t test; n=2.

Having established the capacity for FH to directly interact with either PadA or Hsa, it was then important to investigate if this binding occurred on the surface of *S. gordonii* cells. For these assays, binding by wild-type (WT) *S. gordonii* was compared to a range of knockout mutants: Δ*padA* (UB2864), Δ*hsa* (UB2930) and Δ*srtA* (UB2576). In *S. gordonii*, membrane-associated transpeptidase sortase A (SrtA) is responsible for covalently anchoring a major family of proteins (LPxTG family) to the cell surface, including PadA and Hsa (Paterson and Mitchell, 2004; Davies, Svensäter and Herzberg, 2009). By including the *srtA* mutant (UB2576) in these assays, it was therefore possible to investigate the effect of removing all LPxTG-family proteins from the surface of *S. gordonii*. *L. lactis* MG1363 (UB754) was included in these experiments as a negative control.

Binding of bacterial cells to immobilised FH was detected by crystal violet assay. Measurement of absorbance at 595 nm allowed quantification of bacterial adhesion levels. *S. gordonii* WT was found to bind immobilised purified FH only at low levels. *S. gordonii* Δ*padA* was reduced by 30%, but this was not statistically significant. *L. lactis* was significantly reduced by 52% with an even greater reduction of 73% and 80% seen for *S. gordonii* Δ*hsa* and Δ*srtA* respectively (Figure 3.10). However, as the levels of binding displayed in these studies were extremely low and at the limit of detection for this assay, it was considered that these data should be interpreted with caution. As such, an ELISA technique was also employed to detect the capacity for immobilised bacteria to bind FH from solution. This resulted in high background readings, possibly as a result of non-specific binding by antibody to bacterial cells. As a consequence, this technique was not pursued further (data not shown).

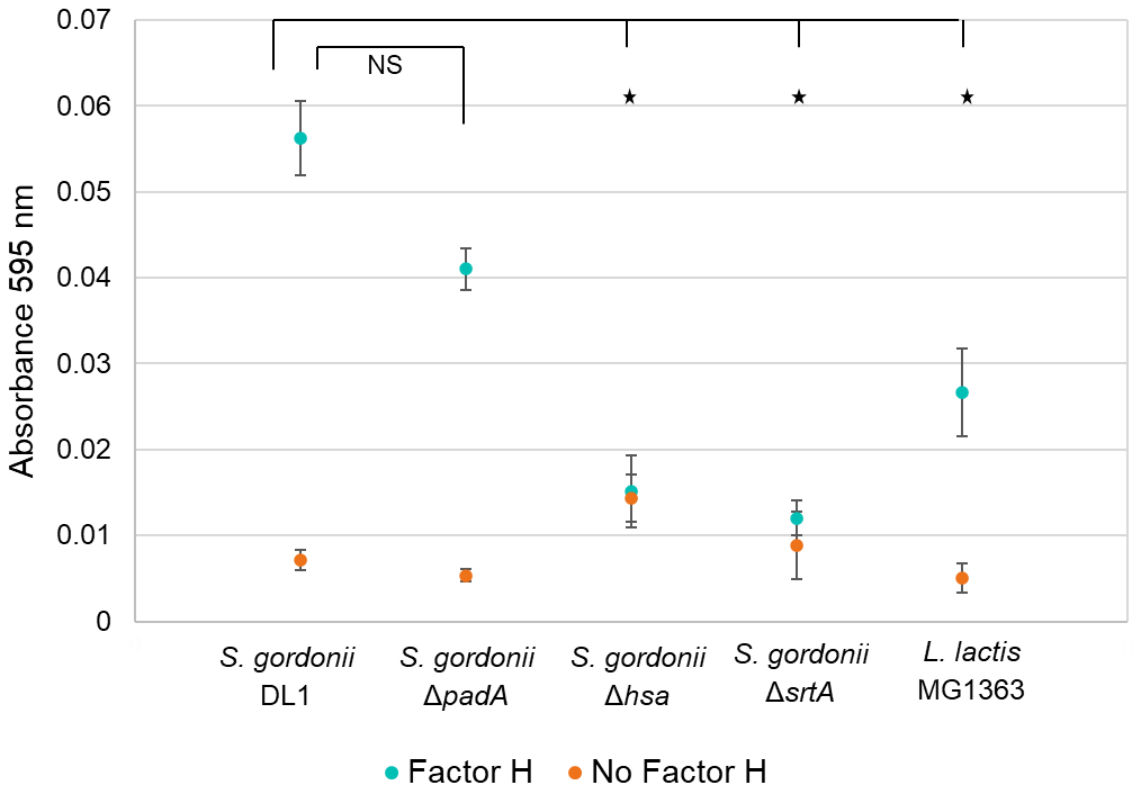

Figure 3.10 Adhesion of *S. gordonii* DL1 WT and isogenic mutants to immobilised FH (20 µg/ml) over 2 h was determined by crystal violet assay. Background readings for wells with no cells have been subtracted. Error bars indicate SEM. **P*<0.05, NS=not significant relative to *S. gordonii* WT as determined by one-way ANOVA and post-hoc Tukey test; n=3.

An alternative approach to investigating the ability of bacterial cells to bind purified FH was adopted, based on the method of Gustafsson *et al.* (2013). Further strains were also included for investigation: the complemented versions of *S. gordonii* Δ*hsa* and Δ*padA* knockout strains and two versions of the Δ*padA* knockout mutant that had been constructed using different mutagenesis approaches: *S. gordonii* Δ*padA::aad9* (UB2723), which is the parent strain for complemented mutant (UB2724), and *S. gordonii* Δ*padA* (UB2864). For the complemented strains, expression of *hsa* or *padA* is under the control of a nisin-inducible promotor. The first step with these investigations was therefore to confirm expression of PadA or

Hsa by the complemented strains when cultured in medium supplemented with 100 ng/ml nisin. Immunodot blot analysis confirmed nisin induction of expression and validation of the knockout mutants (Figure 3.11). The anti-PadA serum showed poor reactivity, which has been previously noted (Haworth, 2015).

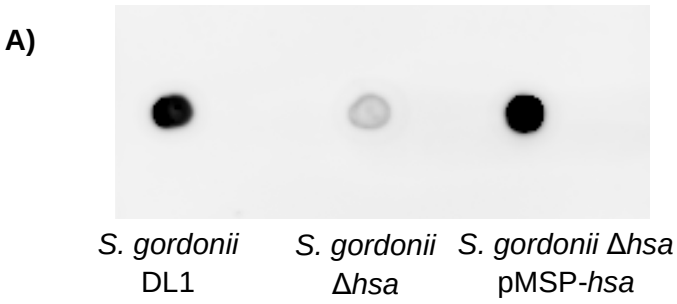

Figure 3.11 Immunodot blot confirming expression of Hsa and PadA by *S. gordonii* knockout and complemented mutant strains. Overnight broth cultures (2 µl) were spotted onto nitrocellulose and allowed to air dry. The presence of Hsa (A) or PadA (B) was detected by immunodot blot analysis using anti-Hsa primary antibody (1:1000) or anti-PadA primary antibody (1:200), respectively, and secondary HRP-conjugated antibody.

Having confirmed their relevant phenotypes, the range of *S. gordonii padA* and *hsa* knockout and complemented mutants, together with Δ*srtA* and *L. lactis* negative controls, were investigated for ability to bind purified FH. Briefly, bacterial cells were incubated in purified FH solution for 1 h at 37 °C, before being washed and the proteins bound to the surface of the bacteria eluted using glycine-HCl solution. Cell-

free eluates were then concentrated ten-fold by centrifugation under vacuum and the presence of FH detected by immunodot blot analysis (Figure 3.12). Purified FH was used as a positive control and showed good reactivity. The S. *gordonii* Δ*padA* knockout mutants UB2864 and UB2723 were reduced by 90% and 50% respectively compared with *S. gordonii* WT. *S. gordonii* Δ*hsa* and Δ*srtA* knockout mutants were both reduced by approximately 80% compared with *S. gordonii* WT. *L. lactis* did not bind FH from solution. Complementation of the Δ*padA* mutant did not restore binding. Complementation of the Δ*hsa* mutant restored binding to a level greater than that of *S. gordonii* WT (110%).

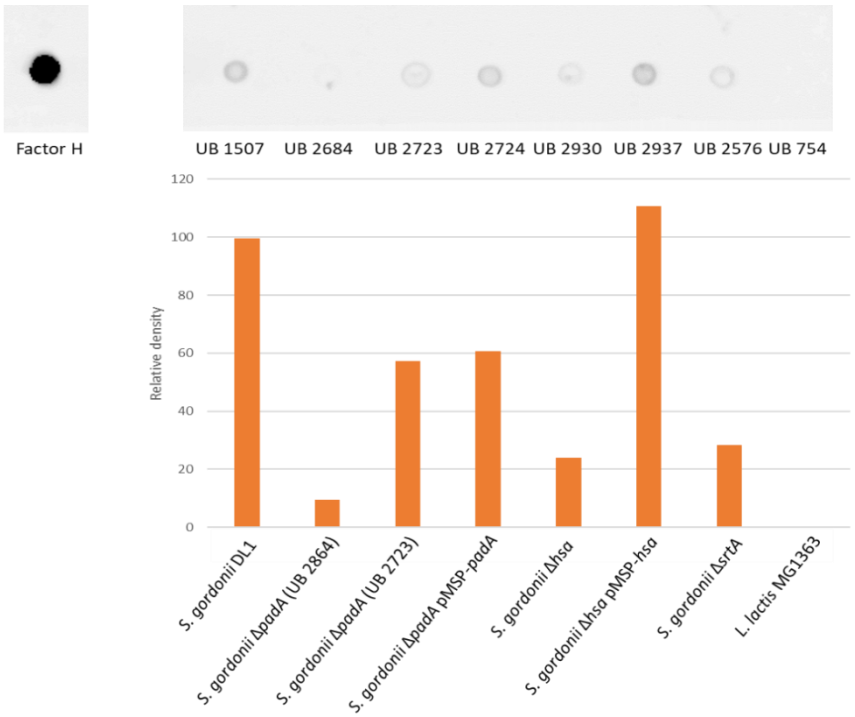

Figure 3.12 Detection of bacterial binding to purified FH by immunodot blot analysis. Bacterial cells were incubated in FH solution. Bound proteins were released using glycine-HCl (method 2.7) and concentrated by centrifugation under vacuum. Concentrated solution was spotted onto nitrocellulose and the presence of FH was detected by immunodot blot analysis. Densitometry analysis was performed using Image Quant TL software.

The glycine-HCl method of eluting bound proteins was then employed to investigate bacterial recruitment of FH from human serum (Figure 3.13). *S. gordonii* Δ*hsa* was 80% reduced in binding to FH compared to WT *S. gordonii*, and complementation restored binding levels to 76% of those of the WT. Likewise, both *padA* knockout strains were reduced in binding (by 96% and 75%), and complementation restored binding to 66% of WT levels. Binding by Δ*srtA* was reduced to 19% of that seen for WT. No binding to FH by *L. lactis* was detected. Purified FH and human serum were used as the positive controls and showed good reactivity. Taken together, these data provide further evidence that *S. gordonii* DL1 can bind FH from serum, particularly via Hsa, and with PadA and possibly other LPxTG-family proteins implicated in this process.

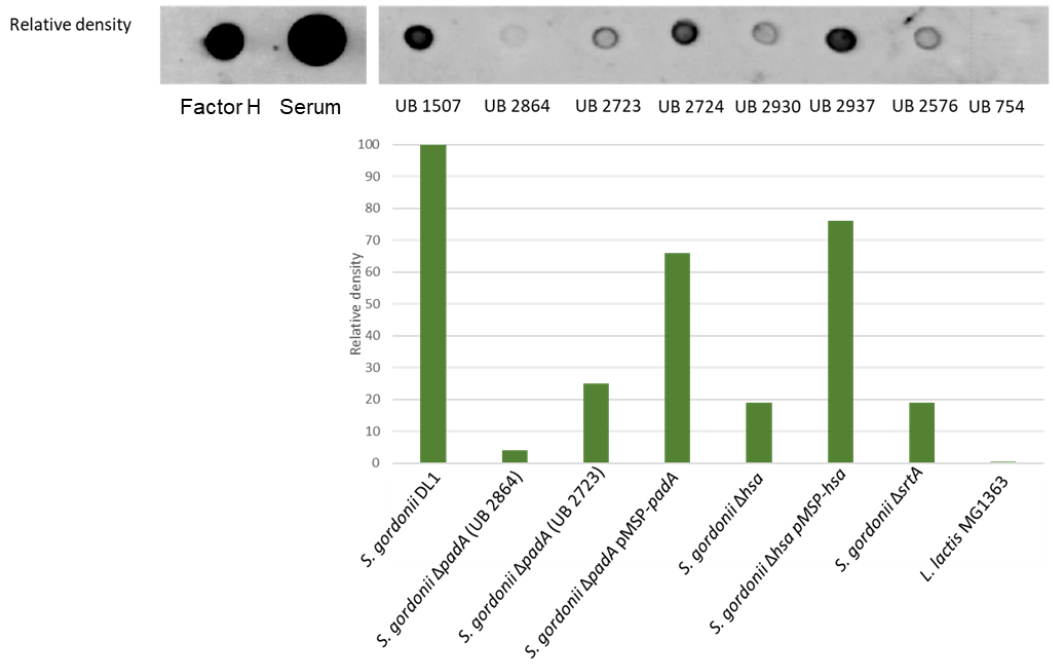

Figure 3.13 Detection of bacterial recruitment of FH from human serum by immunodot blot analysis.Bacterial cells were incubated in human serum. Bound proteins were released using glycine-HCl (method 2.7) and concentrated by centrifugation under vacuum. Concentrated solution was spotted onto nitrocellulose and the presence of FH was detected by immunodot blot analysis. Densitometry analysis was performed using Image Quant TL software.

3.2.1.1. Potential effects of FH engagement on *S. gordonii* survival in blood

Since FH is a negative regulator of the amplification loop in the complement cascade, it was hypothesised that the apparent PadA- and/or Hsa-mediated binding of *S. gordonii* to FH may limit deposition of C3b on the bacterial cell surface. This could, in turn, limit *S. gordonii* opsonisation and recognition by immune system effectors when in the blood stream. To test this hypothesis, *S. gordonii* WT and PadA/Hsa isogenic mutants were investigated for survival in human blood at 2 h. To more robustly assess the potential role of PadA in this process, consideration was first given to the fact that the *padA* gene is part of an operon comprising *padC*, *padA* and *padB*, and so knockout mutants of each of these genes were investigated. The functions of proteins PadC and PadB have not been elucidated to date, but the *padB* gene is predicted to encode a LPxTG-family protein containing G5 domain repeats. For other bacterial proteins, such domains have been associated with promoting binding in the formation of biofilms (Bateman, Holden and Yeats, 2005). The *padC* gene is predicted to encode a thioredoxin signature-like protein, potentially with a role in cytochrome aa_3 maturation (Loferer, Bott and Hennecke, 1993). At 2 h, *S. gordonii* Δ*padA* cells showed a trend of lower survival compared to *S. gordonii* WT. The *padB* and *padC* knockout strains showed more variability, but the *padC* mutant was lower than WT in 2 out of the 3 experiments (Figure 3.14). Due to donor-to-donor variation, these differences were not statistically significant, but for each independent experiment, *S. gordonii* Δ*padA* exhibited lower survival than WT.

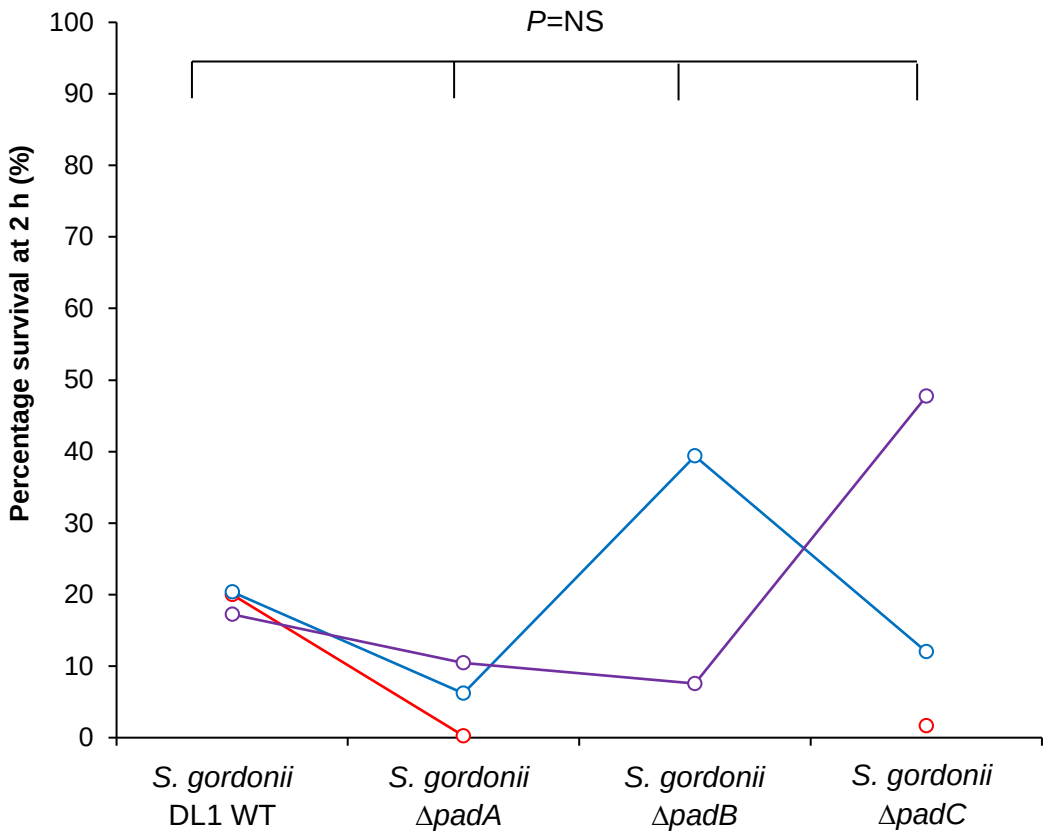

Figure 3.14 Percentage survival of *S. gordonii* WT and *padCAB* mutants incubated in human whole blood for 2 h. Method 2.3. Each coloured line connecting data points indicates data from individual donors. *P*=NS as determined by Friedman test; n=3 (*S. gordonii* Δ*padB*, n=2, as bacteria failed to grow for one experiment)

Survival of *S. gordonii* Δ*hsa* was lower than *S. gordonii* WT on all occasions, while the Δ*srtA* knockout mutant was lower than *S. gordonii* WT for 2 out of the 3 experiments (Figure 3.15). For these studies, an additional knockout mutant of gene *sndA* was also included. SndA (streptococcal nuclease domain A) is a cell wall-anchored LPxTG-family protein of *S. gordonii* with exonuclease activity (Lawler, 2019), and a homologue of this protein had been shown to promote survival of *S. sanguinis* in blood by degrading neutrophil extracellular traps (NETs) (Morita *et al.*, 2014). Survival of *S. gordonii* Δ*sndA* was reduced in comparison to *S. gordonii* WT on 2 out of 3 occasions (Fig. 3.15). Again, due to donor-to donor variation, these

differences were not statistically significant, but *S. gordonii* Δ*hsa* exhibited reduced survival relative to WT for each independent experiment.

Taken together, these data support the hypothesis that PadA, Hsa and possibly other LPxTG family proteins such as SndA promote survival of *S. gordonii* in blood.

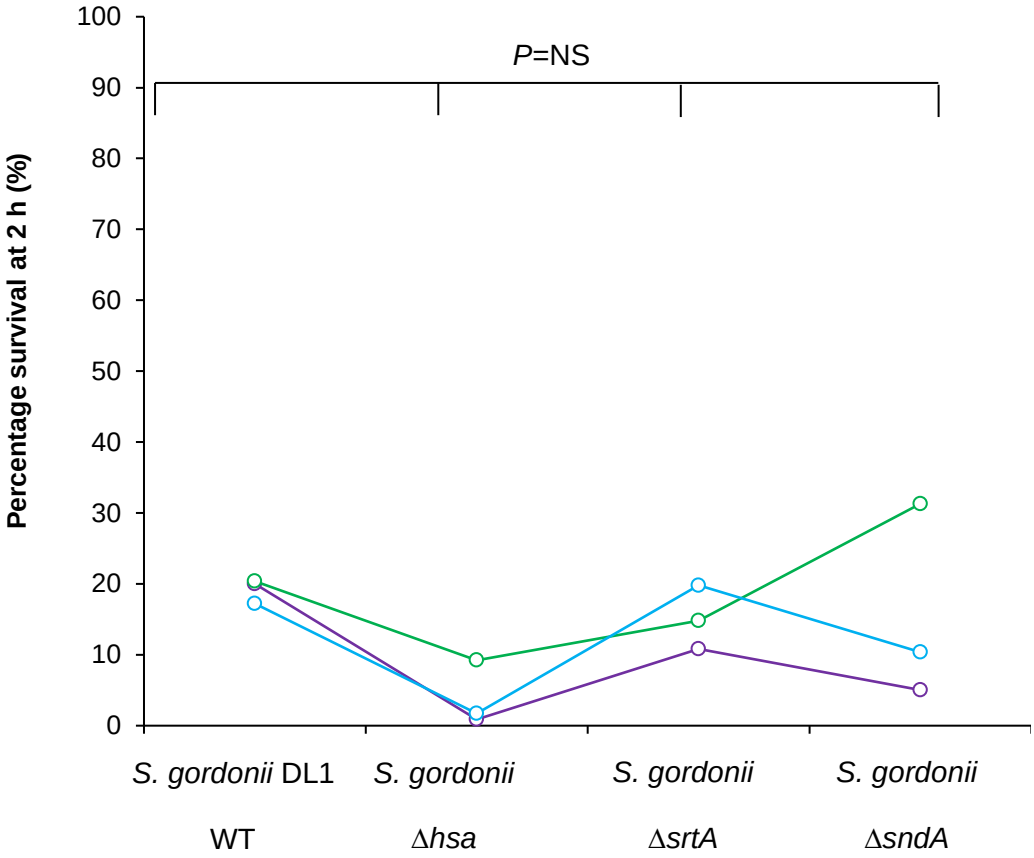

Figure 3.15 Percentage survival of *S. gordonii* WT and adhesin mutants incubated in human whole blood for 2 h. Method 2.3. Each coloured line connecting data points indicates data from individual donors. *P*=NS as determined by Friedman test; n=3.

3.2.2. Interactions with Vn

Vn is a multifunctional protein present in plasma, ECM and in platelet α-granules. Several microorganisms have been noted for their ability to bind Vn and the capacity to exploit this binding to promote survival in blood (Singh, Su and Riesbeck, 2010). Previous work had demonstrated the capacity for *S. gordonii* to bind Vn via a mechanism that involved PadA and Hsa (Haworth *et al.*, 2017a). Since it was possible that this mechanism could enable *S. gordonii* to resist complement-mediated lysis within the blood stream, studies were undertaken to test this hypothesis and to further define the specific molecular interactions of *S. gordonii* with Vn.

Initially, experiments were performed to identify the region(s) of Vn that was targeted by *S. gordonii*. Bacterial adhesion to Vn in the presence of blocking agents against the N- or C-terminal domains of Vn (Figure 3.16A) was determined. A monoclonal antibody raised against the N-terminal region of the Vn molecule significantly impaired binding by *S. gordonii* WT by 26%, as determined by crystal violet assay (Figure 3.16B). The ability of bacteria to bind the C-terminal heparin-binding domain of Vn was investigated by ELISA. ELISA was used due to concerns that access to the C-terminal domain of Vn would be impaired by immobilisation. Attachment of Vn to immobilised *S. gordonii* was significantly reduced by nearly 50% in the presence of heparin relative to control in the absence of heparin (Figure 3.16C). These data imply that *S. gordonii* has capacity to bind both the N-terminal and C-terminal domains of Vn.

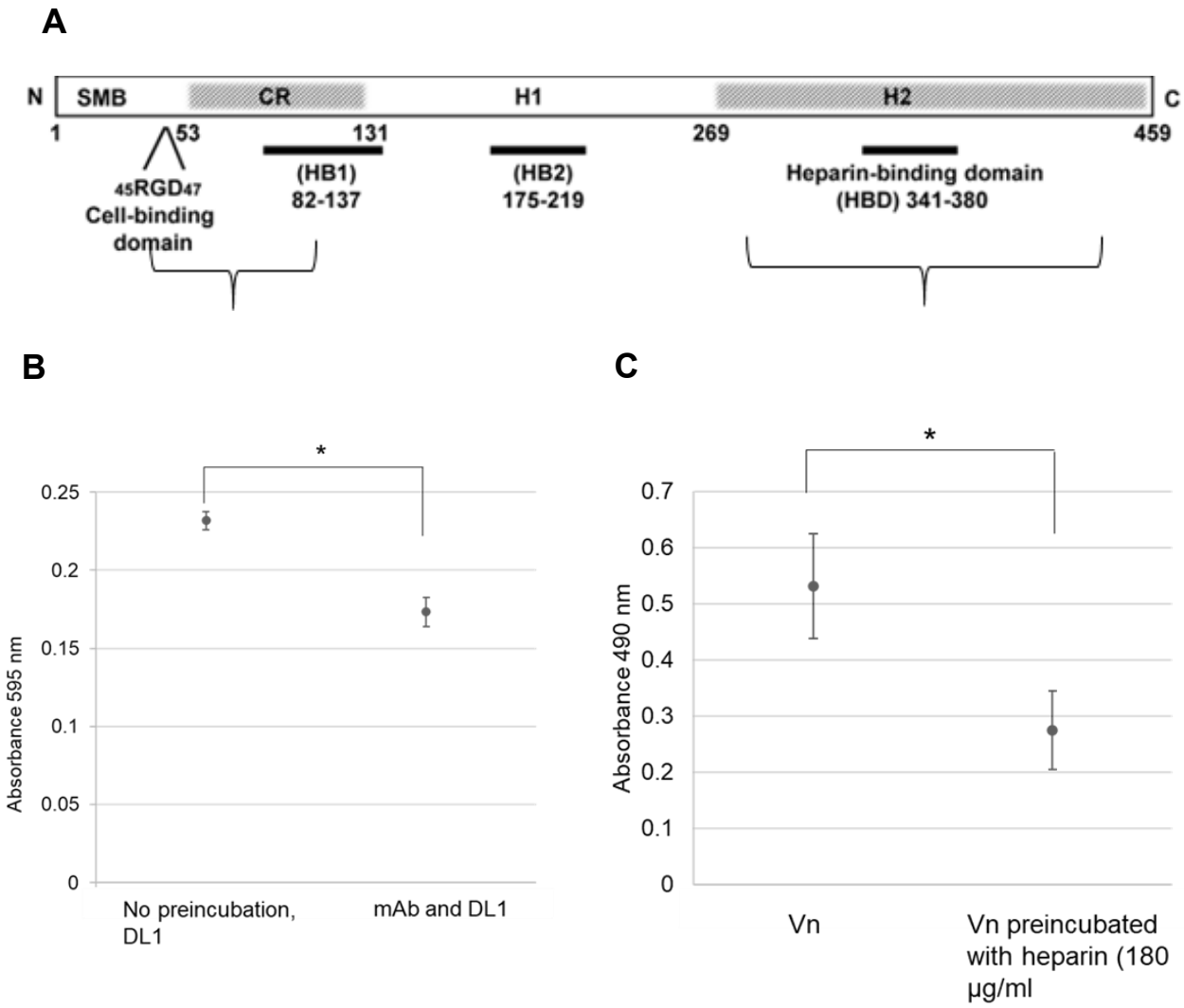

Figure 3.16 Binding of *S. gordonii* to Vn domains

(A) Schematic of Vn domains (adapted from Sa E Cunha *et al.,* 2010), indicating somatomedin B domain (SMB), connecting region (CR), haemopexin domains 1 and 2 (H1, H2), and low affinity heparin binding domains 1 and 2 (HB1, HB2).

(B) Adherence of *S. gordonii* to the N-terminus of multimeric Vn. Multimeric Vn (+/-preincubation with mAb 8E6) was immobilised and adherence of *S. gordonii* WT cells detected by crystal violet assay. **P*<0.05 relative to no mAb control; n=4.

(C) Adherence of *S. gordonii* WT to the C-terminus of multimeric Vn. *S. gordonii* cells were immobilised in microtitre plate wells and incubated with multimeric Vn (+/- preincubation with heparin). Bound Vn was then detected by ELISA assay. **P*<0.05 relative to no blocking agent control as determined by Student's t test; n=4.

Having demonstrated that *S. gordonii* bound Vn at both N- and C-terminal sites, investigations were then performed to explore if binding Vn via PadA and/or Hsa could promote *S. gordonii* survival. This was achieved by testing the effects of serum supplementation with Vn on survival of WT *S. gordonii* compared to Δ*padA* or Δ*hsa* mutants (Figure 3.17). Unlike the effects seen in whole human blood, there was no clear difference of *S. gordonii* Δ*hsa* compared to WT after 2 h in human serum, regardless of the presence of Vn supplementation or not. Survival of Δ*padA* was lower than WT on 2 occasions and comparable or slightly higher than WT on 2 occasions and this was largely unchanged by Vn supplementation.

Together, these data imply that the capacity for *S. gordonii* to bind Vn may promote its survival in human blood and that PadA may be responsible for mediating this effect.

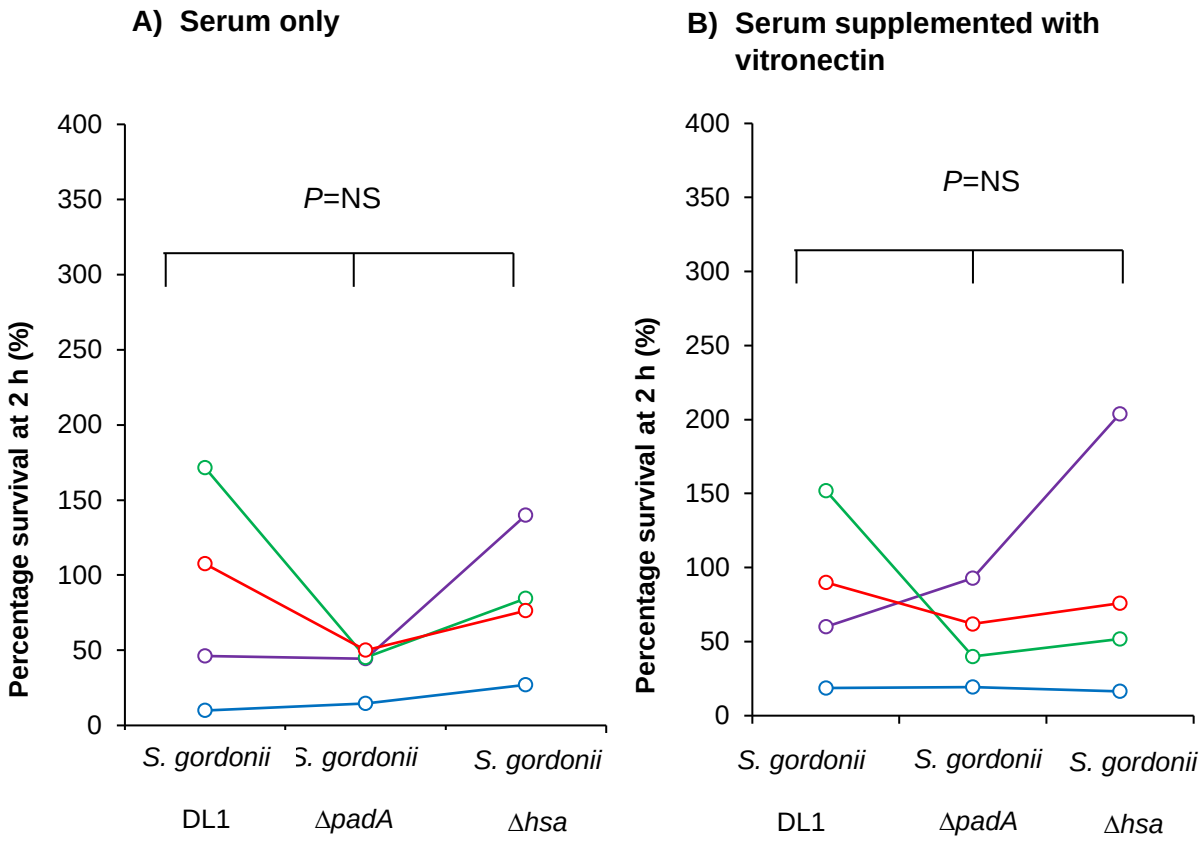

Figure 3.17 Effects of serum supplementation with Vn on percentage survival of *S. gordonii* WT and mutants. Washed bacterial cells were incubated in human serum A) without or B) with supplementation with Vn (1 µg/ml) for 2 h in a candle jar at 37 ℃, 80 rpm. Method 2.3. Each coloured line connecting data points indicates data from individual donors. *P*=NS (not significant) as determined by Friedman test; n=4.

4. Discussion

4.1. Survival of oral *Streptococcus* bacteria in human blood

Bacteraemia is a necessary precursor to platelet interaction and heart valve colonisation in IE pathogenesis. The periodontal and gingival tissues are extremely thin, highly vascular and can be disturbed easily, allowing ingress of bacteria from nearby biofilms (Parahitiyawa *et al.*, 2009). For the bacteria to survive in the bloodstream for any length of time, they need to employ mechanisms to avoid detection by both cellular and humoral components of the host immune system.

The most recent clinical evidence available suggests that post-procedure bacteraemia is dampened down by the host within 12 minutes following a dental procedure (Roberts *et al.*, 2006). In the present *in vitro* study, more WT *S. gordonii* DL1 bacterial cells survived at 2 h in anti-coagulated whole blood, compared to WT *L. lactis* MG1363 cells. *L. lactis* is widely employed as a negative control in studies of Gram-positive bacteria, due to its lack of natural colonisation of human tissues. The difference in percentage survival between *Streptococcus* and the negative *Lactococcus* control bacteria supports the hypothesis that *S. gordonii* has evolved specific mechanisms to prolong survival in human blood. Even after 6 h, a proportion of the *S. gordonii* inoculum survived. Historically, the average circulation time in resting adults was calculated as 13 secs (Tarr, Oppenheimer and Sager, 1933), with the circulation time being defined as the time taken for blood to travel from the arm vein, through the heart and lungs, up to the mouth. Based on the data presented here, this suggests that during bacteraemia, viable *S. gordonii* cells

will have circulated past potentially susceptible heart valve locations numerous times over just a few hours.

S. gordonii was compared with other streptococcal endocarditis pathogens for ability to persist in human blood. As might be expected when blood samples from a range of donors were compared, there was significant biological variability. Nonetheless, of the VGS strains tested, *S. oralis* and *S. sanguinis* strains were comparable to *S. gordonii* in blood persistence. *S. oralis* CR834 has been shown to interact directly with platelets, leading to platelet microaggregate formation, perhaps providing a mechanism for stable septic vegetations on heart valves (Tilley *et al.*, 2013). The derivation of the species name *S. sanguinis* (Latin for blood) indicates its role in the pathogenesis of IE. Platelet glycoprotein GPIb binds *S. sanguinis* directly (Kerrigan *et al.*, 2002), suggesting a role for platelet aggregation in thrombus formation. A *S. sanguinis* cell wall-anchored exonuclease, homologous to SndA in *S. gordonii*, also contributes to survival in blood against NET bactericidal activity (Morita *et al.*, 2014). Survival of the fourth VGS tested, *S. mitis*, was lower than that of *S. gordonii* at 2 h. *S. mitis* is the predominant species isolated from the buccal mucosa in healthy patients (Huttenhower, 2012), but in neutropenic patients, *S. mitis* is more likely than other VGS to enter the bloodstream and cause serious infections (Shelburne *et al.*, 2014). *S. mitis* was also found to have the highest rate of antibiotic resistance when compared with 3 other VGS (Doern *et al.*, 1996). The final *Streptococcus* species tested, *S. salivarius*, is rarely implicated in IE, being identified as the causative agent of only 2% of VGS-associated IE (Felix and Gurunathan, 2014). *S. salivarius* is also rarely isolated from blood cultures and when isolated, has historically often been considered a contaminant. In the studies

presented here, *S. salivarius* exhibited the lowest level of survival in blood across the streptococci tested. Taken together, these data show a general correlation between known association with IE and VGS capacity to survive in human blood, with the exception of *S. mitis*. However, the latter discrepancy could be related to strain-specific variation, which was seen for other *Streptococcus* species tested here. Furthermore, as the IE disease process is multifactorial, other factors including abundance in the oral cavity, ability to bind platelets and other mechanisms for evading host immune responses, will impact overall virulence potential. Nonetheless, it seems logical that persistence in the bloodstream would enhance the opportunity for *Streptococcus* bacteria to colonise the endocardial surface and drive unwanted thrombosis, and therefore IE disease risk. These data also validated the use of *S. gordonii* as the model *Streptococcus* species for further investigating the mechanistic basis for survival in human blood.

4.2. Interaction with complement cascade mediators

A study published during the current project investigated complement opsonisation levels of four VGS: *S. sanguinis*, *S. gordonii*, *S. mitis* and *S. oralis* (Alves *et al.*, 2019). *S. sanguinis* was found to have reduced susceptibility to complement opsonisation, with C3b deposition levels reduced compared to the other species investigated. Complement interaction was also found to be strain-specific, with strains isolated from systemic infections more resistant to complement opsonisation compared to oral isolates of the same species (Alves *et al.*, 2019). There was diversity in C3b/iC3b deposition observed for *S. mitis* and *S. oralis*. It was postulated that CRP binding by phosphorylcholine-containing cell wall components may be critical, with *cps* genes

in *S. mitis* and *S. oralis* responsible for capsule synthesis. Serine-rich repeat adhesins Hsa/GspB (*S. gordonii*) and SrpA (*S. sanguinis*) were hypothesised to have roles in complement deposition. It was suggested that these adhesins could cause bacterial coating with sialic-acid soluble serum components (e.g. fetuin proteins), which would in turn recruit FH (Alves *et al.*, 2019). This was supported by preliminary data from the Oral Microbiology group at Bristol that demonstrated the capacity for *S. gordonii* to bind serum components FH and Vn via adhesins PadA and Hsa, functions that may promote bacterial complement evasion. These potential immune evasion mechanisms were therefore investigated further in this project.

4.2.1. Factor H

The current investigation utilised a native form of PadA that had been expressed and secreted by *S. gordonii*, rather than with recombinant forms from *E. coli* used in previous studies. This provided greater confidence that PadA would adopt its regular conformation and carry any post-translational modifications such as glycosylation. The histidine tag also allowed immobilisation of PadA via its C-terminus, mimicking the orientation of this adhesin when expressed on the bacterial cell surface. In this way, direct binding between purified FH and $PadA_{His6}$ immobilised on Ni^{2+}-microtitre plate wells was demonstrated, possibly implicating FH-binding sites in the unattached N-terminus of PadA. Similar FH binding was also shown using Hsa_{His6}, indicating that both adhesins have the capacity to bind directly to FH. These functions were then further verified using *S. gordonii* bacterial cells. The data imply that both PadA and Hsa have the capacity to bind FH directly, but it appears Hsa may be primarily responsible for mediating this effect in human blood.

Binding to FH was significantly diminished for the *srtA* knockout mutant, supporting the role of LPxTG-family adhesins in this process. Nonetheless, binding to FH was not ablated. The residual binding could be due to adhesins that are transiently held within the bacterial cell wall, as has been reported previously (Nobbs *et al.*, 2008; Turner *et al.*, 2009). Alternatively, other non-LPxTG family surface determinants of *S. gordonii* may be able to mediate binding to FH at a low level. Levels of binding for the *hsa* and *srtA* knockout mutants were comparable.

Microbes can protect themselves from complement-mediated killing by recruiting host FH. In conjunction with factor I, FH mediates degradation of C3b, a key driver of the complement cascade. A range of microbes have been found to bind FH at a common site, located on one side of domain 20 of FH (Meri *et al.*, 2013). Binding here is thought to generate a tripartite complex comprising microbial protein:FH:C3b, the formation of which increases FH-mediated inactivation of C3b. Consequently, as seen for Sbi of *S. aureus* (Haupt *et al.*, 2008) or PspC of *S. pneumoniae* (Janulczyk *et al.*, 2000), it is possible that PadA/Hsa-mediated binding to FH may limit deposition of C3b on the surface of *S. gordonii* and so promote its survival within the bloodstream. This hypothesis is supported by the reduced survival of *S. gordonii padA* and *hsa* knockout mutants at 2 h in human blood compared to *S. gordonii* WT (Figures 3.14, 3.15).

Hsa is a sialic acid binding adhesin and as such, might be expected to mediate interactions with sialylated surfaces in the bloodstream. FH has specific sialic acid binding domains (SCRs 16-20) (Ram *et al.*, 1998). Therefore it is possible that Hsa interacts with FH via sialylated serum proteins, such as fetuin, as postulated by

Alves *et al*., 2019. This may provide an explanation as to why Hsa may have a dominant role in human blood over PadA. Both PadA and Hsa can bind FH directly, but bridging with serum proteins may predominate in blood. The relative strength of such a binding interaction over other available sialylated proteins in the blood is an important consideration for future studies. Both SrpA, the serine-rich repeat adhesin homologue in *S. sanguinis*, and Hsa selectively target intact bacteria to sialylated ligands on platelets within human whole blood over the excess of alternative sialoglycan ligands in soluble and cellular components of human blood (Deng *et al.*, 2014). Less is known regarding potential FH binding targets for PadA. However, further information was determined regarding the potential role of the *padCAB* operon. There was no evidence that LPxTG-family protein PadB is involved in promoting *S. gordonii* survival in blood, but decreased survival was seen for 2 out of the 3 experiments for the Δ*padC* mutant. PadC is a thioesterase, and thiol oxidoreductases have been shown in other Gram-positive bacteria to stabilise adhesin structures (Reardon-Robinson *et al.*, 2015a; Reardon-Robinson *et al.*, 2015b). It is therefore possible that PadC may be required to ensure that PadA adopts the correct conformation.

Other *S. gordonii* mutants were also investigated in the current study for survival in blood, namely *srtA* and *sndA*. The *srtA* knockout mutant was reduced on 2 out of 3 occasions in survival in comparison to WT. While a more significant impairment might have been predicted, again this could reflect the activity of transiently expressed LPxTG-family proteins or other non-LPxTG family surface determinants. A reduction in survival was seen for the Δ*sndA* mutant also on 2 out of 3 occasions. As shown for *S. sanguinis* homologue SWAN, this may indicate the capacity for

exonuclease SndA to promote *S. gordonii* survival by facilitating escape from NET-mediated killing. This proposed mechanism of neutrophil evasion warrants further investigation.

4.2.2. Vitronectin

As well as its effects on fibrinolysis, coagulation and plasminogen activation following tissue injury, Vn regulates the lytic pathway in the complement system. Vn can inhibit C9 polymerisation and bind C5b-7, preventing this complex (C5b-7 complex or membrane attack complex [MAC]) from inserting into bacterial cell membranes (Milis *et al.*, 1993). Several microorganisms have therefore been shown to bind Vn and thereby block complement components, conferring serum resistance (Singh, Su and Riesbeck, 2010). Heparin-binding region 3 within the C-terminus of Vn is postulated to be a common bacterial binding site (Singh, Su and Riesbeck, 2010). *N. meningitidis* has been shown to bind sulphated tyrosine residues in the N-terminal domain (Sa E Cunha, Griffiths and Virji, 2010), while inhibition of C9 binding and pore formation occurs at the heparin-binding region (Milis *et al.*, 1993). Since *S. gordonii*-Vn binding capabilities, mediated by PadA and Hsa, had recently been demonstrated both with *S. gordonii* knockout mutants and purified protein (Haworth *et al.*, 2017a; Lohiya, 2018), experiments were performed to determine the *S. gordonii* binding site on Vn. Inhibition of bacterial binding to Vn by addition of mAb 8E6, which binds an epitope in the N-terminus (sulphated tyrosine residues at positions 56 and 59), suggests that *S. gordonii* can directly interact with this N-terminus region, similar to *N. meningitidis* (Griffiths *et al.*, 2009). There was also evidence that *S. gordonii* binds a C-terminal heparin-binding

site in Vn, in common with many other microorganisms (Singh, Su and Riesbeck, 2010), since the presence of heparin impaired attachment of *S. gordonii* to Vn by 50%. It therefore seems that *S. gordonii* may be capable of mediating interactions with more than one domain of Vn, reflecting the complex multifunctional nature of this plasma protein. Further work is required to define if this reflects the targeting of different domains of Vn by PadA and Hsa.

Through binding Vn, it is possible that *S. gordonii* is able to protect itself against complement-mediated killing in blood, or by evading immune recognition by coating itself in host molecules. However, *S. gordonii* WT serum resistance was not increased by the addition of Vn to serum in this study. One explanation for this finding could be that sufficient Vn was already present in the non-supplemented human serum to mediate interactions with the bacteria. *S. gordonii* Δ*hsa* showed no clear difference in survival compared to the WT in non-supplemented human serum. This result was in stark contrast to the whole blood survival assays, where Δ*hsa* was nearly ablated. Serum lacks any platelets or cells compared to the whole blood. This suggests that Hsa may mediate the bulk of its protective effects by interaction with cellular components in the bloodstream. *S. gordonii* Δ*padA*, in contrast, was reduced compared to WT on 2 out of 4 occasions and comparable or slightly increased compared to WT on 2 out of 4 occasions. Vn supplementation had no clear effect on survival. These data imply that PadA plays a protective role in blood survival. Further elucidation of the relative roles of PadA and Hsa in serum resistance may require use of double mutants, with complementation of each protein in turn.

In addition to complement inhibition, Vn binding by *S. gordonii* may facilitate attachment to damaged endothelial tissue, which is abundant with Vn (Haworth, 2015). Host cell integrin interaction with the N-terminus of Vn would allow the C-terminus to bind bacteria and this cross-linking may be a crucial part of endothelium colonisation in IE. Association of *S. gordonii* cells with human coronary artery endothelial cells is increased in the presence of serum, suggesting that a serum protein is implicated in endothelial-bacterial cell bridging (unpublished data). The presence of Vn at exposed ECM sites in the oral cavity is also likely to play a role in bacterial attachment and biofilm formation. Interestingly, significant increases in Vn (as well as fibronectin and laminin) were found in the periodontal ligament of teeth that had undergone orthodontic tooth movement in a human *in vivo* study (Morales-González *et al.*, 2010). As periodontal and gingival tissues are disrupted during separator placement and other orthodontic procedures, the increased expression of these ECM proteins may be a critical mechanism for streptococcal ingress into the bloodstream.

4.3. Hsa-like and PadA-like proteins

PadA and Hsa interact with human platelets and are implicated in biofilm formation within the human body. As such, these two adhesins are likely to be major virulence factors in the pathogenesis of *S. gordonii* IE. In further support of this, data presented in this project indicate that PadA and Hsa both bind FH and Vn, which may promote survival in blood. Unpublished data suggest that C4BP may also interact with PadA and Hsa (Lohiya, 2018). C4BP is another regulatory protein that modulates the classical and lectin pathways of the complement system, and can act

as a bridging molecule to facilitate *S. pyogenes* adhesion to and invasion of endothelial cells (Ermert *et al.*, 2013). Given their critical role in IE pathogenesis, the distribution of PadA- and Hsa-like adhesins across other potential IE pathogens is therefore of interest.

PadA homologues are found in a range of streptococci and in *Abiotrophia defectiva* (Table 4.1). *Streptococcus cristatus* has a protein with 99.79% identity to PadA. This microorganism is another VGS member. Descriptions of *S. cristatus*-associated endocarditis are rare, but this may well reflect difficulties in phenotypic identification of this species. A PadA-like protein, FmtB, is also found in *S. oralis* NCTC 11427. This strain was used in this project (UB2178) and found to exhibit high survival levels in human blood relative to *S. gordonii*. Indeed, the fact that PadA-like proteins have been identified in most of the species investigated in this project, but not *S. salivarius*, suggests that PadA may be conserved amongst a number of *Streptococcus* species associated with IE, where it can promote survival in blood.

Table 4.1 A selection of bacterial proteins with high homology to *S. gordonii* DL1 PadA retrieved from BLASTP online database
(https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 24/10/19)

Species	Protein	Percentage identity (%)*
Streptococcus cristatus	Isopeptide-forming domain-containing fimbrial protein	99.79
Streptococcus sp. 2_1_36FAA	Isopeptide-forming domain-containing fimbrial protein	98.05
Streptococcus sp. HMSC10A01	Isopeptide-forming domain-containing fimbrial protein	86.70
S. sanguinis	Cell wall surface protein	69.94
S. mitis	Isopeptide-forming domain-containing fimbrial protein	63.67
S. oralis subsp. *tigurinus*	Cell surface protein	62.94
S. parasanguinis	Isopeptide-forming domain-containing fimbrial protein	61.76
Streptococcus rubneri	Isopeptide-forming domain-containing fimbrial protein	59.19
S. oralis ATCC 35037 / NCTC 11427	FmtB protein	58.05
Streptococcus sp. LYSM12	Isopeptide-forming domain-containing fimbrial protein	52.05
Streptococcus respiraculi	Isopeptide-forming domain-containing fimbrial protein	51.60
Abiotrophia defectiva	Hypothetical protein	41.84

*****Percentage identity describes the percentage of amino acid residues with a direct match in alignment to the entire *S. gordonii* PadA amino acid sequence.

Hsa homologues are presented in Table 4.2. GspB of *S. gordonii* M99 is a homologue of Hsa. GspB orthologues have previously been found to be prevalent, but not ubiquitous, amongst oral streptococcal strains (Takamatsu *et al.*, 2005). All of the streptococcal species investigated in this project, which have also been

associated with bacteraemia following separator placement, have proteins with homology to the Hsa protein of *S. gordonii* DL1. *S. pneumoniae* is a highly invasive pathogen implicated in pneumonia, septicaemia and meningitis and also displays a Hsa homologue. *Aerococcus viridans*, another microorganism with an Hsa homologue, is a causative agent of IE (Pien *et al.*, 1984).

Table 4.2 A selection of bacterial proteins with high homology to *S. gordonii* DL1 Hsa, retrieved from BLASTP online database
(https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 24/10/19)

Species	**Protein**	**Percentage identity (%)***
S. gordonii M99	GspB	96.35
S. sp AS14	Accessory Sec-dependent serine-rich glycoprotein adhesin	75.86
S. sanguinis	Accessory Sec-dependent serine-rich glycoprotein adhesin	70.14
S. mitis	Serine-rich repeat glycoprotein	55.56
S. pneumoniae	Accessory Sec-dependent serine-rich glycoprotein adhesin	52.94
S. oralis	Accessory Sec-dependent serine-rich glycoprotein adhesin	51.69
S. salivarius	Accessory Sec-dependent serine-rich glycoprotein adhesin	49.58
Aerococcus viridans	LPXTG cell wall domain-containing protein	31.00

*****Percentage identity describes the percentage of amino acid residues with a direct match in alignment to the entire *S. gordonii* Hsa amino acid sequence.

Taken together, the presence of both PadA and Hsa homologues across a range of streptococcal and other bacterial species that are implicated in IE supports the hypothesis that these proteins play an important role in the pathogenesis of this

disease, and therefore could represent suitable therapeutic targets for the prevention of IE.

4.4. Mechanisms for *S. gordonii* survival in blood

The known and proposed interactions of *S. gordonii* with the human complement system are summarised in Figure 4.1. *S. gordonii* binds Vn and FH, negative regulators of C5b-9 and C3b deposition, respectively. PadA and Hsa may also bind C4b, thereby inhibiting the classical and lectin pathways. As well as avoiding complement detection, evasion of macrophages by *S. gordonii* is possible through resistance to ROS and delaying the maturation of phagosomes (Croft *et al.*, 2018). Data in this project (Figures 3.15, 3.17) support the idea that Hsa may confer its protective effects in the bloodstream by preferentially interacting with cellular components. Such resistance of bacteria to cellular killing by the host is also likely to be an important virulence determinant of IE (Young Lee *et al.*, 2006). Bacterial aggregation and activation of platelets under flow conditions in the bloodstream means that platelets are also implicated in promoting bacterial survival in blood and the progression of IE, with the platelets acting as “Trojan Horse” carriers of bacteria to sites of injury (Deng *et al.*, 2014).

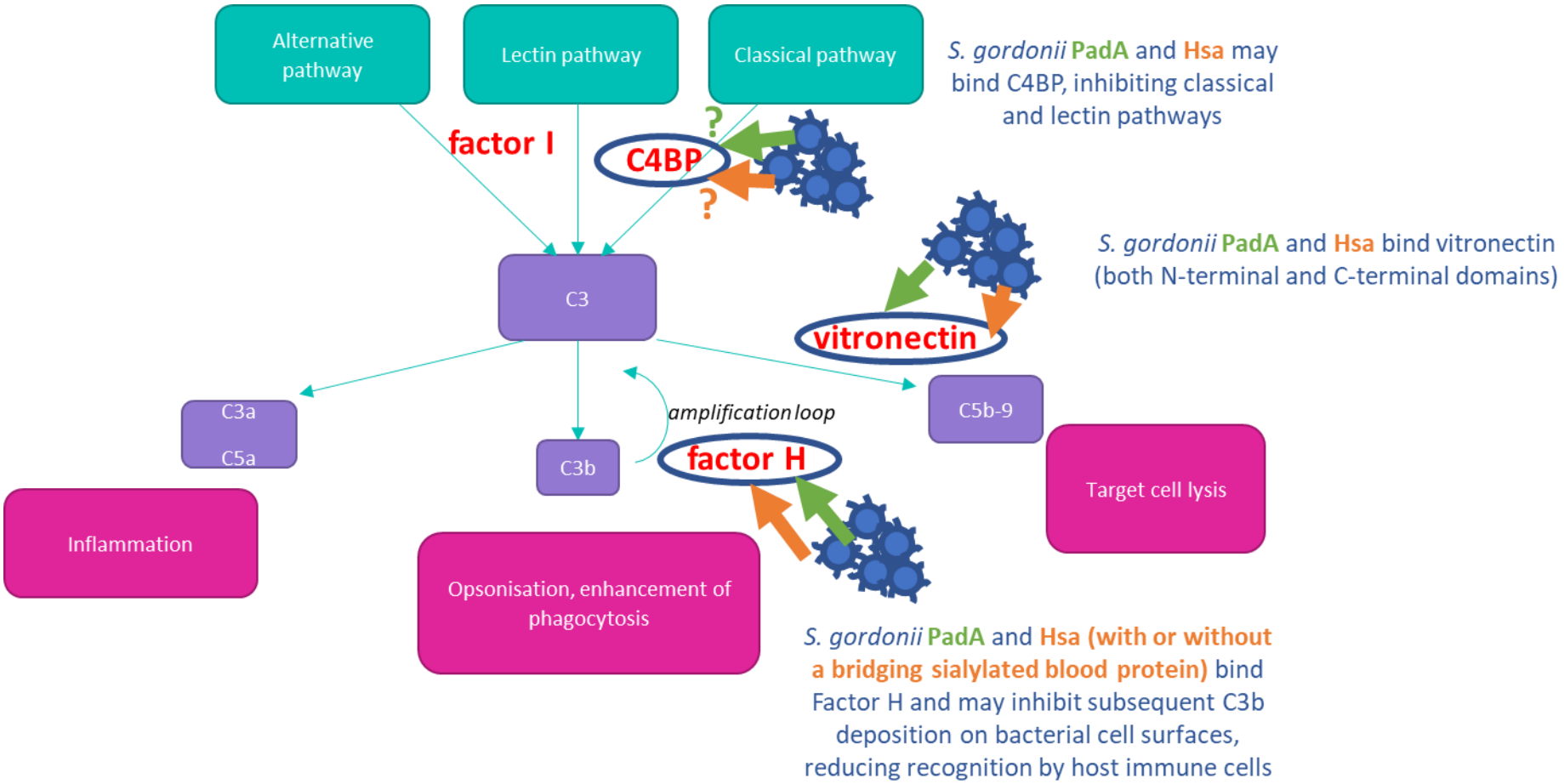

Figure 4.1 Summary of interactions between *S. gordonii* and the complement system. Negative regulators of the complement system are shown in red.

4.5. Revised model for streptococcal IE pathogenesis

Despite significant shifts in the epidemiology of IE in recent years, with an observed increase in staphylococcal endocarditis in new risk groups, it is still important to understand streptococcal IE pathogenesis. IE is increasing in the UK, in keeping with global trends. In a recent study of a rural UK population, streptococci were the predominant cause of healthcare-associated IE, in contrast to other areas of the world where staphylococci are the main cause (Hughes, Epstein and Prasad, 2019). A revised model for streptococcal IE pathogenesis in the context of orthodontic procedures is depicted in Figure 4.2. In summary, *S. gordonii* colonises dental hard surfaces and exposed ECM surfaces in the oral cavity. Upregulation of Vn expression during orthodontic tooth movement may aid *S. gordonii* colonisation. Orthodontic procedures, such as separator placement, induce bacteraemia, with the bacterial cells in the bloodstream recruiting both Vn and FH, evading complement-mediated killing. Direct binding of bacterial cells to platelets causes infected microthrombi to form. Bacterial survival in the bloodstream then allows streptococcal colonisation of damaged endocardial surfaces via putative Vn and FH bridging mechanisms, causing infected vegetations to form on the surfaces of heart valves.

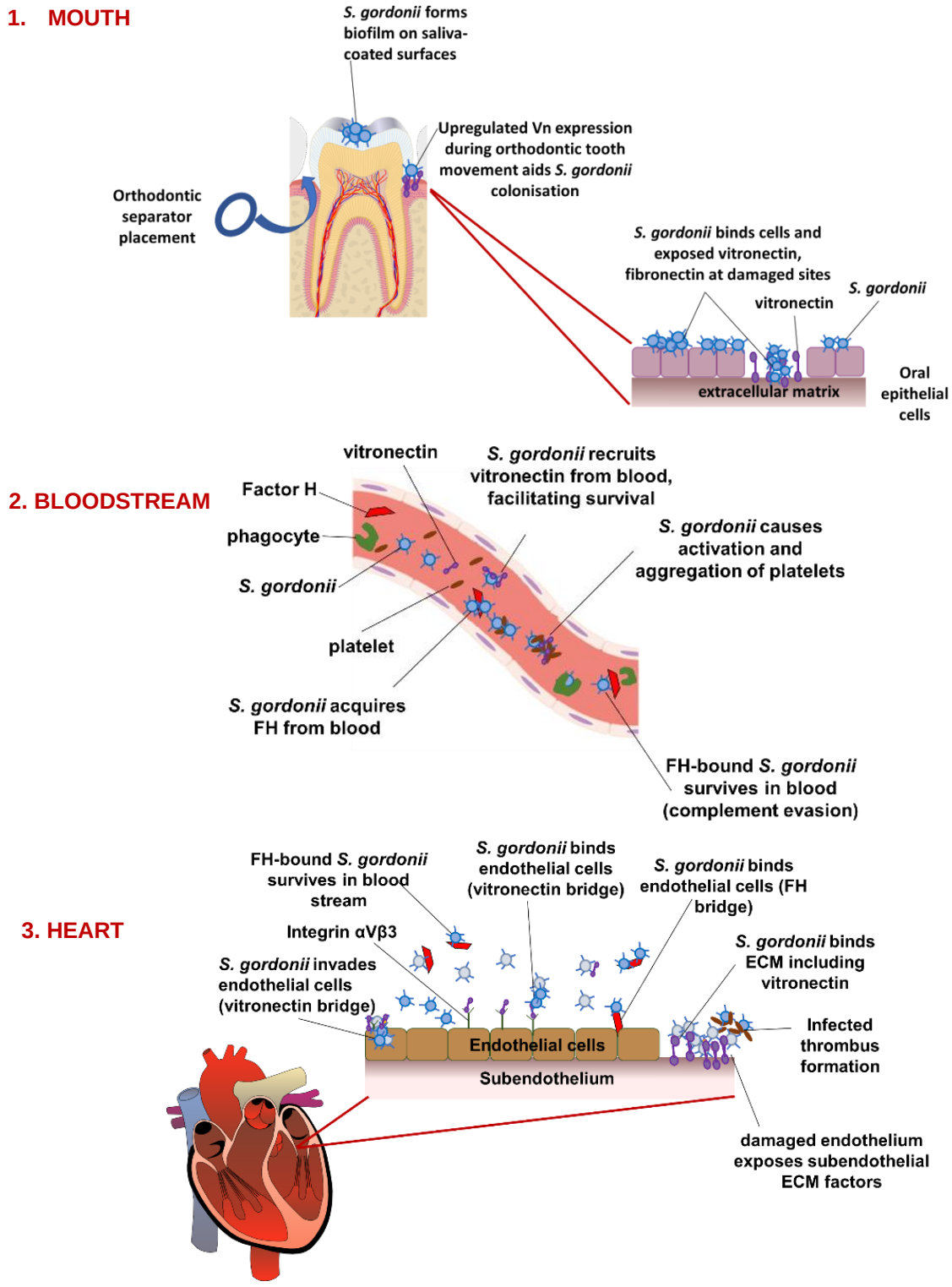

Figure 4.2 Model for the pathogenesis of orthodontics-associated streptococcal IE from mouth to heart.

4.6. Clinical implications

The clinical diagnosis of IE is complex. The standardised Duke-Li criteria (Li *et al.*, 2000a), based upon the use of both imaging and microbiology, form the basis of IE diagnosis, but there are limitations to this classification (Iung and Duval, 2019). Molecular microbiological techniques are therefore likely to form the basis of future diagnostic techniques, due to their sensitivity and specificity (Faraji *et al.*, 2018). For this, the ability to differentiate between streptococcal endocarditis pathogens is important, because clinical outcomes and disease progression are likely to differ between species and strains. In the current project, not only did the time course of survival of different streptococci vary, but so did the resistance to complement deposition between bacteraemia-associated strains compared to oral isolates (Alves *et al.*, 2019). This project therefore highlights the need for a better understanding of virulence across the oral streptococci.

The mechano-transduction that occurs during orthodontic tooth movement enables remodelling of periodontal ligament tissues, allowing the desired tooth movement to occur. This conversion of biomechanical stimuli into intracellular biochemical stimuli is complex. ECM changes as a result of tooth movement result in integrin clustering and activation and signal transduction into cells, stimulating intracellular signalling pathways (Feller *et al.*, 2015). Based on former studies and the data presented here, significant increases in expression of ECM components, such as fibronectin and Vn, during orthodontic tooth movement (Morales-González *et al.*, 2010) may be predicted to facilitate colonisation of damaged gingival tissues by oral streptococci that bind

these ECM components. The orthodontic patient may therefore be inherently more at risk of developing bacteraemia from oral bacteria such as *S. gordonii* and thus potentially at greater risk of IE.

The orthodontic profession is in a unique position, having regular access to patients at ages when they are most likely to be developing IE as a child. The orthodontic team must be aware of the causes, signs and symptoms of IE and promote preventive advice where possible. Despite being rare, the consequences of developing IE are devastating. It may be necessary to re-visit clinical guidelines for the prevention of IE in the future.

Mechanisms for streptococcal survival in the bloodstream, such as those identified in this project, could be targeted for the prevention or treatment of IE in the future. Novel inhibitors, perhaps applied topically at the site of separator placement, for example, could be targeted against known bacterial-host binding interactions to prevent bacteria using the immune system to their advantage. This would promote host clearance of bacteria from the blood stream and thus reduce IE disease risk. This approach could also negate the need for antibiotic usage, which would be beneficial in the current climate of growing antibiotic resistance.

4.7. Conclusions

Key findings are:

1) A range of streptococcal species are linked to bacteraemia following orthodontic separator placement.
2) Blood survival rates are species- and strain-dependent, but there is a general correlation between association of species with IE and persistence in blood.
3) *S. gordonii* PadA and Hsa bind FH of the complement system. It is postulated that interaction primarily by Hsa enables bacterial cell evasion of the host immune system.
4) *S. gordonii* binds Vn at both N-terminal and C-terminal sites via PadA and Hsa. This represents a second mechanism via which *S. gordonii* may evade the host immune system, primarily by PadA.
5) Hsa-mediated bacterial survival in blood may be more related to cellular interactions than PadA-mediated survival.
6) Hsa-like and PadA-like proteins are found in a range of *Streptococcus* and other bacterial species associated with IE and therefore represent potential targets for the prevention or management of IE.
7) The orthodontic profession should be vigilant regarding the risks of bacteraemia associated with orthodontic procedures, especially considering the changes in ECM composition during active tooth movement that could promote bacteraemia with VGS.

4.8. Future work

i) Determination of the relative importance of Hsa and PadA in serum resistance

The use of double *hsa* and *padA* knockout mutants, with complementation of each protein in turn, would be invaluable for determining the relative importance of these proteins in serum resistance and potential binding to C3b.

ii) Evidence of PadA/Hsa complex formation

Both Hsa and PadA cell-surface proteins have been implicated in colonisation, biofilm formation, platelet binding and serum resistance. Co-localisation studies with fluorescent probes would enable detection of Hsa/PadA complexes on bacterial cell surfaces.

iii) Investigation of FH as a bridging molecule for host interactions

FH is thought to mediate host cell-microbe interactions, with FH acting as a bridging molecule (Jozsi, 2017). Bacterial adhesion of human cardiac endothelial cells could be investigated in the presence of serum depleted in or supplemented with FH.

iv) Determination of the molecular mechanism for Vn binding by Hsa and PadA

Further work is required to identify domains of Vn targeted by Hsa and PadA. This could be achieved by examining the effects of blocking agents on *padA* and *hsa* knockout mutants.

v) Further evidence of *S. gordonii* neutrophil evasion

SndA contribution to survival in blood via evasion of NET-mediated killing needs further investigation.

vi) Clinical perspectives

The effect of upregulated ECM protein expression in the PDL during orthodontic tooth movement on bacterial colonisation and entry into the bloodstream should be investigated further. New diagnostic procedures or clinical monitoring techniques could be developed based on Hsa/PadA antibodies. Ultimately, better understanding regarding the evasion of host defences by streptococci in the bloodstream could lead to the development of novel strategies for the prevention or treatment of IE.

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